Vol. XIII, No. 2 July, 1919

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WILLIAM WESLEY & SON, 28, Essex Street, Strand, London, W.C. 2
CHICAGO: The University of Chicago Press
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TORONTO: J. M. Dent & Sons, Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1919

Price Ten Shillings and Sixpence net

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XII. NOTE ON XEROPHTHALMIA IN RATS.

By ELSIE CHARLOTTE BULLEY.

(*Received March 3rd, 1919.*)

Among the diseases which have been attributed to a deficiency in the fat-soluble *A* accessory substance is a condition of the eyes now described as xerophthalmia. McCollum, Simmonds and Pitz [1918] indeed speak of this relation in very definite terms. "Xerophthalmia and polyneuritis are abundantly demonstrated to have their origin in the lack of a sufficient amount of the fat-soluble *A* and water-soluble *B* respectively in the diet." That eye symptoms in rats may occur as the result of deficiencies in the diet seems to be common experience. It is very important, however, to distinguish between symptoms due directly to a specific dietetic error and any that may be due to infection, encouraged, may be, by diminished resistance due to the diet.

My own experience seems to point to the fact that so far as xerophthalmia is concerned the latter relation is the true one. In considering this question I have taken absence of normal growth as evidence of a deficient ration.

It is desirable at the outset to view the origin and growth of the idea that xerophthalmia is due to the absence of some essential nutritive factor and later of the view that fat-soluble *A* is the pivot on which the matter turns.

Osborne and Mendel [1914, 1] in writing of their protein-free milk state that the addition of butter fat to this substance avoids failure in growth and "an infectious eye disease." They repeat this statement [1914, 2, 1915] when, dealing with the question of separating the essential fat factor, mention is again made of the fact that animals fed with an inefficient fat fraction developed purulent eyes. McCollum and Simmonds [1917] cite eye disease as a possible deficiency disease due to absence of the fat factor, and the writers quote cases of children developing it on fat-deficient diets. As previously mentioned [1918] they definitely state that xerophthalmia is brought about by absence of the fat factor. Osborne and Mendel [1918] make a further allusion to the condition stating that on a fat deficient diet the symptoms occurred, and were cured by the addition of liver oil to the food. In a recent paper by Harden and Zilva [1918] eye symptoms are again taken as indicative of the absence of fat-soluble *A*.

In January, 1913, a series of experiments was carried out in this laboratory solely with a view to clearing up this matter of eye disease, which had made its appearance amongst animals then being fed by Professor Hopkins on synthetic diets in which the mineral supply was Osborne and Mendel's artificial

salt mixture. The results of these experiments have never been published, and it is with Professor Hopkins' permission that I allude to them here. He found that animals fed on extracted starch, extracted caseinogen, sugar, salts, and lard developed an ulcerative condition of the cornea with conjunctivitis within 14 days from the commencement of the experiment, identical with that present in animals fed, in previous experiments, on a similar artificial diet, though these earlier rats had been fed with a different end in view. In the more extreme cases there was complete dissolution of the eyeball with rupture of the cornea. Many of the animals died, but one or two survived and on being given 3 cc. milk as an addendum to the diet, the eye symptoms cleared up. This led to the assumption that the disease was due to some deficiency in the diet which the 3 cc. of milk supplied. In 1915 Dr Ginsaburo Totani, at the suggestion of Professor Hopkins, undertook some experiments based upon these previous findings with the intention of making a thorough histological study of the eye lesions. The experiments were not done in the same building as were the earlier ones, but other conditions were precisely similar. The weight of the animals fell as usual, but no eye disease at all was obtained with purified artificial diets identical with those previously used, and after many weeks the experiment was given up.

Save in the case of the experiments done by Hopkins I have no detailed information as to the similarity of the symptoms observed by the different workers, nor as to the rapidity with which the xerophthalmia develops. In Hopkins' 1913 experiments, as already stated, it appeared as early as the fourteenth day. It is perhaps legitimate to assume that in the other cases cessation of growth, or initial absence of growth, marked the point at which the condition of the eyes became pathological, and that on resumption of growth the eye disease abated and ultimately cleared up. This, at any rate, seems to be the correct conclusion to draw from the evidence available. My own experience in the matter which has led me to the view already expressed is the following:

Since January, 1918, to the present date I have had approximately 500 young rats on varying experimental diets. To all these diets has been added water-soluble *B* in the form of a fat-free alcoholic extract of a yeast preparation. About 50 per cent. of the animals have received a supply of fat-soluble *A*, the remaining 50 per cent. having been on diets where fat-soluble *A* has been either deficient or absent. As regards this latter statement a diet has been considered "deficient" in fat-soluble *A* when young rats, receiving a proper supply of the water-soluble accessory substance, have failed to grow after, and often long before, the eighth week of the experiment. They have been supposed completely lacking in the factor when lard has been the source of fat supply. The basal diet of all foods fed has consisted of extracted caseinogen, unextracted starch, sugar and salts. The stock animals from which the experimental animals have been selected have been fed on bread and milk and oats, with the addition for the last four months of various vegetables.

During the whole of the period five cases only of xerophthalmia have occurred. Two of these were rats which were being fed on margarine with which was incorporated an unknown quantity of egg yolk as the source of fat-soluble *A*; one animal was on basal diet plus lard plus 2 cc. of milk per day; another was being given fatty acids in place of fat, and the remaining rat was a stock animal which had never been on any diet save the normal stock food.

The two first-mentioned animals were given butter as an addendum to their diet when the disease was well established, and their eyes were treated daily with boracic lotion. Both animals became cured within a week. Their growth curve was poor before the addition and good after. The animal having 2 cc. milk daily did not clear up at all and ultimately died; the one on fatty acids as its source of fat was fed with an addendum of butter and began to get better after a week. (This animal is still under observation and is not completely cured yet.) The rat on stock diet was a considerable time recovering—some five or six weeks—but did so ultimately. Thus of the five cases,

Two occurred on a diet where fat-soluble *A* was supplied, if to a limited extent.

One occurred on a diet where fat-soluble *A* was presumably present in sufficient quantity.

One occurred on a diet where fat-soluble *A* was probably absent.

One occurred on an ample stock diet.

Again,

Two animals were growing moderately well.

One animal lost in weight.

One animal neither declined nor increased in weight.

Each experiment lasted some three or four months and in each experiment there were at least eight other animals on the respective diets, showing no symptoms at all. Xerophthalmia on this evidence would appear to have no direct relation to diet, since these five animals were on different types of food; and to have little relation to growth.

It may be objected that the basal diet was not entirely free from fat-soluble *A* or that the fats used contained a sufficiency of the essential factor to prevent the onset of the disease. In view of the fact that practically all the animals on fat-soluble *A* deficient diets, though having an abundant supply of water-soluble *B*, failed ultimately to grow normally, and in many cases to grow at all, the former factor, if there at all, must, according to current views, have been present only in negligible quantities.

In addition to the above experience there remains that of Totani, who was using more highly purified foods and always lard as his source of fat. I consider that the discrepancy between Hopkins' and Totani's results, in which the animals were upon a diet absolutely identical in each case, is due to there having been the infection present in the former case and not in the latter, and that the freedom of my own animals from this disease is owing

(1) to the fact that no trouble has been spared to keep the animals under the very cleanest and healthiest conditions, and

(2) that care has always been taken to treat very promptly with boracic lotion any cases of slightly sore-looking eyes.

As regards the clearing up of the symptoms, which the quoted workers have observed on the addition of fat-soluble *A* it is surely probable that, as in so many cases of obstinate infection, general improvement of health brings about a cure of the local condition. I feel convinced that with avoidance of initial infection, experimental animals can be kept almost entirely free from this so-called deficiency disease, whether fat-soluble *A* be present or absent, and that it is dangerous to draw conclusions as to the fat-soluble *A* content of any diet from the appearance of xerophthalmia.

REFERENCES.

Harden and Zilva (1918). *Biochem. J.*, **12**, 408.
McCollum and Simmonds (1917). *J. Biol. Chem.*, **32**, 181.
—— Simmonds and Pitz (1918). *J. Biol. Chem.*, **33**, 413.
Osborne and Mendel (1914, 1). *J. Biol. Chem.*, **16**, 423.
—— (1914, 2). *J. Biol. Chem.*, **17**, 401.
—— (1915). *J. Biol. Chem.*, **20**, 379.
—— (1918). *J. Biol. Chem.*, **34**, 17.

XIII. THE PREPARATION OF SILICA JELLY FOR USE AS A BACTERIOLOGICAL MEDIUM.

By ALBERT TOM LEGG.

University College, Reading.

(*Received March 6th, 1919.*)

During the course of experiments involving the cultivation of seedlings in nitrogen-free media, a supply of silica jelly was required, free from combined nitrogen and of such consistency that it would support the growth of seedlings in tubes.

Preparation of the jelly according to published methods [Smith, 1905] failed to give satisfactory or uniform results, especially with regard to the consistency of the gel, although great care was taken at every stage of the preparation. Since other workers seem to have experienced similar difficulties, it was thought that an attempt to standardise the method of preparation of silica jelly might prove useful and possibly save much labour. The present note is the outcome of such an attempt and is published in the hope that it may be of service to other workers.

Preliminary work showed that the critical stages occurred during dialysis and that it was necessary to work with membranes of permeability varying within certain limits in order to obtain a uniform gel after autoclaving.

Collodion membranes, prepared and graded, as described by Brown [1915], were tried for dialysing, but it was subsequently found easy to prepare membranes of the requisite permeability without alcoholic treatment.

A solution of collodion is prepared by dissolving 6 g. of clean white guncotton in 100 cc. of a mixture of equal parts of absolute alcohol and ether. A quantity of not less than 500 cc. is made up and is allowed to stand for at least 24 hours to dissolve. The solution is then decanted into a bottle of convenient size and shape for holding in the hand. Should minute insoluble particles be noticed on the side of the wetted flask, due to unequal nitration of the guncotton, the solution is decanted through a filter of glass wool. These insoluble particles are the cause of fragile and leaky membranes. Filtered collodion is always more satisfactory provided that precautions are taken to prevent undue loss of ether by evaporation. It is impracticable to prepare a solution of collodion of definite composition, but for the purpose of making membranes it should be of a consistency similar to that of a heavy oil.

Test tubes $7\frac{1}{2} \times 1\frac{1}{2}$ inches were found most serviceable for the work. They must be thoroughly clean and dry, and also smooth; tubes with projections on the glass give rise to leaky membranes.

A test tube is held in a slanting position in the hand and slowly rotated while the collodion is poured down the inside until it is completely coated and a depth of about one inch has collected at the bottom. The collodion is then poured *slowly* back into the bottle, the tube being rotated as before. As soon as all the mobile collodion has entered the bottle, the tube is quickly inverted and its mouth placed firmly on a piece of paper with a folded duster, or pad of some kind, underneath, so that the tube is completely closed and an ethereal atmosphere maintained inside while it is allowed to drain for exactly 6 seconds. The accumulation of collodion is quickly wiped off and the membrane rapidly dried by passing a glass tube, attached to a filter pump, up and down the test tube, which is rotated horizontally, meanwhile, to keep the coating of collodion evenly distributed. During this drying process the finger is lightly pressed against the collodion a little way inside the mouth of the tube every eight or ten seconds. Immediately the collodion ceases to adhere to the finger the test tube is quickly and carefully filled with water, and allowed to remain full for at least three minutes to prevent further loss of ether.

It is important that the drying process should not be carried too far; the object in view being to obtain a thick membrane of low permeability; it may take any time from 30 seconds to 4 minutes according to the temperature and humidity of the laboratory.

The membrane is now detached from the mouth of the tube by the aid of a needle and removed by allowing water to pass between it and the glass aided by gentle pressure of the fingers.

These membranes are slightly milky in appearance, *i.e.* of the ether rather than the alcohol type, and are sufficiently strong for easy manipulation. They must be kept under water and never allowed to become dry.

For the dialysis of silicic acid a membrane of this size, when filled with distilled water and suspended in air, should not allow the water to drop oftener than once in twelve seconds. Membranes have been successfully used, with little difference in the time taken for dialysis, which had a range of from 1 drop in 12 seconds to 1 drop in 80 seconds; but the thicker membranes are more reliable and may be used many times.

Pure re-fused sodium silicate was used for the preparation of nitrogen-free media. This is supplied in opaque lumps which are slowly soluble in boiling water, but are readily brought into solution when raised to a temperature of 140° for one hour in the autoclave. 100 g. of sodium silicate in a litre of distilled water is readily diluted to give a solution of specific gravity 1·09.

A standard solution of re-distilled hydrochloric acid of specific gravity 1·10 is now prepared. It requires approximately 530 cc. of distilled water to the litre of acid to reduce the concentrated acid to this density.

Equal volumes of the acid and silicate solutions are taken, the former is poured into a conical flask and the latter into a large separating funnel and dropped into the acid which is shaken meanwhile. This acid mixture is allowed to stand from three to four hours, according to the temperature of the laboratory, in order that the necessary chemical reaction may take place, the longer period being given when the temperature is below 15°. The mixture is then poured into a separating funnel and thence into the membrane, which is supported in a vessel of water to avoid undue strain. When nearly full the neck of the membrane is drawn together and secured with a rubber band and the membrane suspended in a gas cylinder—or other suitable vessel—of distilled water to dialyse. The distilled water is changed hourly during the day and dialysis continued until the silicic acid is neutral to litmus. This usually takes two days. After the first twelve hours the water in the dialyser is tested with litmus paper before each change, which gives an indication of the state of acidity of the solution inside the membrane and avoids unnecessary manipulations.

Running tap water as supplied to the town of Reading was repeatedly tried for dialysis, but always caused the silicic acid to gel in the membrane before dialysis was complete.

When neutral to litmus, the silicic acid is poured into a beaker, the nutrient salts added in concentrated solution, and the medium boiled for five minutes to expel air. If not sufficiently boiled the plugs will be blown out of the tubes in the autoclave and an uneven gel will result. The medium is "tubed" whilst hot and immediately autoclaved for 20 minutes at from 135° —140°.

Silicic acid prepared in this way forms a gel on autoclaving; if slants are required the tubes must be placed in the requisite positions in the autoclave as the gel once formed is irreversible.

The success of the process depends upon:

(1) The use of a membrane of standard permeability. A rather thick membrane of low permeability gives the best results.

(2) A sufficiently long period being given for the sodium silicate and hydrochloric acid to react after mixing. This is a critical factor.

(3) The use of distilled water for dialysing. Constant changes of water especially in the earlier stages of dialysis in a vessel just large enough to take a membrane, are best.

(4) Tubing the medium as quickly as possible after removal from the membrane and immediate autoclaving. The autoclave should be heated up while the medium is being tubed.

If it is not convenient to tube and autoclave the medium immediately after dialysis, it may be kept for about 24 hours in the dialyser. If allowed to stand for a short time in a beaker after boiling, dialysed silicic acid will probably form needle-shaped crystals.

It should be noted that compounds of silicon form deposits on glass.

These deposits are difficult to remove. It is therefore advisable to restrict the use of glass apparatus within reasonable limits during the preparation of the jelly.

In conclusion I wish to thank Dr M. C. Rayner for suggesting this work, and also for giving me much valuable advice and criticism.

REFERENCES.

Brown (1915). *Biochem. J.*, **9**, 591.
Smith, E. (1905). *Bacteria in relation to Plant Diseases*, **1**. 37.

XIV. ELECTRICAL CONDUCTIVITY AS A MEASURE OF THE CONTENT OF ELECTROLYTES OF VEGETABLE SAPS.

By DOROTHY HAYNES.

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(*Received March 7th, 1919.*)

In the course of some work involving a comparison of various methods of extracting saps, it has been found necessary to give attention to conductivity measurements as a means by which the content of electrolytes of samples of sap might be compared. A considerable amount of recent work in plant-physiology has been based on determinations of this nature in which it has been generally assumed that the conductivity of saps can be employed as a direct, though possibly somewhat approximate, measure of the content of electrolytes. It is clearly of importance that this assumption should not be made without adequate investigation, but hitherto no such investigation appears to have been carried out. On the other hand there are grave reasons for denying the validity of the proposition, even as a first and very rough approximation except under certain strictly limited conditions. Attention was first called to this point by an examination of the results of the conductivity measurements made by Dixon and Atkins [1913] on fruit juices. These were found to be uniformly low and the observers point out that this is the case even where acid fruits are in question, the juices of which are known to contain large quantities of organic acids. They explain their results as a consequence of the relatively slight dissociation of organic acids, but this explanation cannot be accepted as sufficient, as will be shown in detail below. It therefore became necessary to examine the matter further.

Since all fruits contain considerable quantities of sugars, it was thought possible that the presence of these in the juices might lower the conductivity and thus account for the discrepancies observed. This supposition was confirmed by comparative determinations of the conductivities of certain electrolytes (potassium chloride and citric acid) in aqueous solution and in solutions containing glucose, from which it was evident that the reduction of conductivity is considerable and must be of importance in the case of sugary saps.

It was subsequently found that the influence of non-electrolytes on the conductivity of aqueous solutions of various electrolytes had been worked out in detail by Arrhenius [1892] for a number of typical substances—electrolytes and non-electrolytes—and the object of the present paper is to discuss the bearing of the results thus obtained on the determination of conductivity in plant juices, especially in relation to the estimation of the content of electrolyte as carried out by Dixon and Atkins [1913].

Arrhenius found that for concentrations of non-electrolytes up to 10 per cent. (by volume) the conductivity of a solution may be expressed by the equation

$$C = C_0\left(1 - \frac{ax}{2}\right)^2$$

where

C = conductivity of the solution containing x per cent. (by volume) of non-electrolyte;

C_0 = conductivity of an aqueous solution of the same electrolyte at the same concentration, and a = a constant.

The constant a varies with the nature of the non-electrolyte and of the conducting solution. It increases with the concentration of electrolyte but the variation is very small for highly dissociated substances. Arrhenius distinguishes the following groups of electrolytes. In each group the value of a is approximately constant for a given non-electrolyte.

Group 1. Strong univalent acids and alkalies.

Group 2. Salts giving univalent anions and cations.

Group 3. Salts giving univalent cations and bivalent anions.

Group 4. Salts giving univalent anions and bivalent cations.

Multivalent acids and weak univalent acids usually give still higher values of a and the same is true of salts which only undergo slight dissociation, *e.g.* $HgCl_2$. It is to be noticed that if $\frac{a^2}{4}$ which is very small be neglected

$$ax = \frac{C_0 - C}{C_0}.$$

The decrease in conductivity is therefore proportional to the concentration of non-electrolyte.

It has been already remarked that the concentration of a highly dissociated electrolyte can be varied within wide limits without producing more than a slight variation in the value of a. It is therefore impossible to find a general explanation of the reduction of conductivity produced by the addition of non-electrolytes by assuming a change in the degree of dissociation and this conclusion is supported by other considerations. Arrhenius attributes the greater part of the reduction to the increase of viscosity produced by the replacement of water by non-electrolyte, and he shows that for any given non-electrolyte a may be expressed with fair accuracy as a linear function of its viscosity in 1 per cent. solution. There is, however, some divergence due to change in the degree of dissociation and in the case of slightly dissociated substances

this factor cannot be neglected. It is to be noticed that the constants of the equation connecting a with the viscosity of the solution vary with the nature of the non-electrolyte. It is, therefore, impossible to find a general formula expressing C_0 in terms of C—the measured conductivity—and the viscosity.

The following examples taken from Arrhenius' tables will give some idea of the values obtained for a.

Table I. *Values of* $a \times 100$ *for various non-electrolytes.*

Non-electrolyte	Group 1	Group 2
Methyl alcohol	1·62	1·75
Ethyl alcohol	1·88	2·34
Isopropyl alcohol	2·03	2·56
Ether	1·63	1·99
Acetone	1·56	1·62
Cane sugar	2·44	2·99

Table II. *Values of* $a \times 100$ *for electrolytes of various constitution.*

Electrolyte	Ethyl alcohol	Cane sugar
Group 1 ...	1·88	2·44
„ 2 ...	2·34	2·99
„ 3 ...	2·51	3·34
„ 4 ...	2·39	3·09
H_2SO_4 $N/4$ ...	2·55	—
H_2SO_4 $N/200$ ...	2·53	2·93
Oxalic acid $N/8$ ...	2·61	3·03
„ „ $N/100$	2·10	2·81

These values represent the percentage decrease in conductivity in a 1 per cent. (by volume) solution of non-electrolyte. By dividing by the density of the non-electrolyte, a more convenient value, a, is obtained which represents the percentage decrease of conductivity in a solution containing 1 g. of non-electrolyte in 100 cc. Table III gives values of a.

Table III. *Values of* $a \left(= \frac{100\,a}{d}\right)$ *for electrolytes of various constitution.*

Electrolyte	Ethyl alcohol	Cane sugar
Group 1 ...	2·38	1·54
„ 2 ...	2·96	1·88
„ 3 ...	3·18	2·10
„ 4 ...	3·03	1·94
H_2SO_4 $N/4$ ...	3·23	—
H_2SO_4 $N/200$ ...	3·20	1·84
Oxalic acid $N/8$ ...	3·30	1·91
„ „ $N/100$	2·66	1·77

d for ethyl alcohol = 0·79. d for cane sugar = 1·59.

It is now possible to estimate the correction which must be made in solutions containing non-electrolytes in order to obtain the corresponding conductivities in aqueous solution from the observed conductivities. Arrhenius'

equation may be taken as accurate up to 10 per cent. (by volume) of non-electrolyte. At 20 per cent. the error in the ratio $\frac{C}{C_0}$ is 4 per cent. Therefore in the case of plant juices the reduction of conductivity may without serious error be taken as proportional to the content of non-electrolytes. For cane sugar solutions the value of a is approximately 2; and glucose produces a very similar effect; the conductivity of a solution containing 5 per cent. of sugar is therefore reduced by 10 per cent. and the factor for the correction of the measured conductivity is $\frac{100}{90} = 1{\cdot}11$. Since, however, many saps contain sugars and other non-electrolytes in much greater concentration than 5 per cent. the correction in these cases may be very large. The magnitude of this effect appears to have been much underrated in various recent researches on the conductivities of vegetable saps even where it is not altogether ignored. The work of Dixon and Atkins is a case in point, and it is interesting to calculate the conductivities of aqueous solutions corresponding to the saps investigated by them in cases where an approximate estimate can be made of the nature and quantity of the non-electrolyte present. For this purpose the values of C may be taken from a table compiled by Atkins [1916, p. 157] from results obtained by him and Dixon. They are shown below in Table IV compared with the corresponding values of C_0.

Table IV.

Fruit	Concentration of sugar assumed %	$C \times 10^5$	$C_0 \times 10^5$
Citrus aurantium	9	208	254
Citrus limonum	2	345	359
Lycopersicum esculentum	4	457	497
Pyrus malus	12	161	212
Vitis vinifera	14·5	112	158

Vaccinium oxycoccus has been omitted, as no estimate of its sugar content could be obtained. The sugar content of the other fruit juices is taken in round numbers from Wehmer's *Pflanzenstoffe* and C_0 is calculated from the equation

$$C_0 = \frac{100\,C}{100 - x},\ a \text{ being taken equal to 2.}$$

The sugar content of fruits is of course very variable, therefore no approximation to accuracy can be claimed for the above figures; they serve however to show the order of magnitude of the error which must be produced by the sugars present in the juices; this amounts in the case of grapes to 40 per cent. It must be remembered too that the sugars are not the only source of error; the effect of alcohols, esters, pectins, dextrins and the remaining non-electrolytes other than sugars which are present has been ignored in the above calculation. In some cases these must play an important part, and a further

examination of one or two of the foregoing results may throw light upon this point besides providing criteria by means of which one may judge certain conclusions which Dixon and Atkins have drawn from their observations.

These conclusions are as follows: (1) that the influence of organic salts and acids on the conductivity is small [1913, p. 439] and (2) that "even in acid fruits by far the larger porportion of the osmotic pressure is still due to non-electrolytes" [Atkins, 1916, p. 157].

These statements as they stand are obviously difficult of acceptance and in the case of (2) the underlying fallacy is evident. Dixon and Atkins have made without further enquiry an assumption which is expressed by Atkins as follows: "that by finding the freezing point of solutions of some standard substance, such as potassium chloride, which have conductivities equal to those of the plant juices under examination, and by subtracting these values from the freezing points of the saps as actually observed, it is possible to get a very approximate measurement of the proportion of the osmotic pressure due to non-electrolytes. The values thus obtained are probably somewhat too low owing to the increased viscosity of the sap occasioned by the sugars in solution" [Atkins, 1916, p. 149]. This is in effect to assume a rough proportionality between osmotic pressure and electrical conductivity for which no theoretical basis exists, osmotic pressure being determined by the joint concentration of molecules and ions while electrical conductivity is a function of the number and velocity of the ions alone. How far astray the assumption may lead can be shown by examining the values obtained by Dixon and Atkins for lemon juice. Lemon juice is stated by Wehmer to contain 5 to 10 per cent. of citric acid; therefore the lemon juice examined probably represented at least a normal solution (6·4 g. in 100 cc.), the freezing point of which has been found to be $-0{\cdot}72°$ whereas by Dixon and Atkins' method the depression of the freezing point due to all the electrolytes in lemon juice works out at 0·170°. Indeed since the value of Δ for lemon juice was found by Dixon and Atkins to be 1·089°, and that for the acid it contains exclusive of salts has been shown to be of the order 0·7 it is clear that the depression of freezing point due to non-electrolytes must be very small. The conclusion stated above must, therefore, be reversed in this case. In lemon juice only a very small part of the osmotic pressure can be due to non-electrolytes.

Only a small part of this error can be attributed to the lowering of the conductivity by viscosity; the error is the natural result of the assumption that equi-conducting solutions of such substances as citric acid and potassium chloride contain an equal number of molecules and ions. In the case of a mono- or di-basic organic acid the error would probably be greater still. Where salts are in question the matter may be somewhat improved, since the degree of dissociation is rather more comparable with that of potassium chloride, but large differences still remain, and added to these is the effect of widely

divergent ionic mobilities, for in this case there are no mobile hydrogen ions present to compensate for the slowly moving anions. There is therefore no theoretical justification for the assumption and as the basis of an empirical formula it is far too precarious to be of any value even in the most limited field.

The conclusion drawn by Dixon and Atkins that the influence of organic salts and acids on the conductivity is small is based on the low conductivities which they observed. It has been shown above that these low results are to some extent a consequence of the presence of non-electrolytes in the saps, but in highly acid saps another very important factor must also be allowed due weight. It is well known that the hydrogen ion concentration of an acid solution may be greatly lessened by the addition of a salt of the same acid. In the case of organic acids possessing heavy slowly moving anions the conductivity is very largely due to hydrogen ions and in consequence the conductivity of a mixture of salt and acid may be less than that of the acid alone, especially where acid salts are formed. Thus the conductivity of a decinormal solution of citric acid was found to be 183×10^{-5} at 25°; that of a similar solution which also contained $N/50$ sodium citrate was found to be 172×10^{-5}, while $N/100$ sodium citrate reduced the conductivity to 142×10^{-5}.[1]

In these solutions where the principal electrolyte is an organic acid and where the degree of dissociation has been reduced by the presence of salts the action of non-electrolytes upon the conductivity becomes less marked and the value of α may fall very considerably below 2. The value of C_0 as calculated above will then be too high. The *joint* influence of organic salts and acids on the conductivity may thus under these conditions become relatively small and the assumption made by Dixon and Atkins be correct; but even where salts exert their maximum effect in reducing conductivity, the measured conductivity will still be very considerably below that of a corresponding aqueous solution if any considerable quantity of non-electrolyte is present.

It must also be observed that the values of α given by Arrhenius were in most cases determined for highly dilute solutions. These values tend to rise as the concentration of the electrolyte is increased, especially where, as for organic acids, the degree of dissociation of the electrolyte is largely reduced by increased concentration. This is an effect apart from and opposite to that produced by salts. Thus it will be seen from Table III that the effect of ethyl alcohol on the conductivity of oxalic acid is increased 24 per cent. when the concentration of the acid rises from $N/100$ to $N/8$. In order to ascertain the value to which α rises in highly concentrated solution, observations have been

[1] Since writing the above attention has been directed to two papers which bear upon this point. Haas [1917] gives measurements of actual and total acidity for a number of plant juices. Hempel [1917] examines the buffer effects produced by salts in the saps of various plants and the relation of P_H to titration in lemon juice.

made of the reduction of conductivity produced in solutions of normal citric acid by the addition of cane sugar and ethyl alcohol respectively. The solutions were made from ordinary commercial citric acid, equal volumes of 2*N* solution being diluted to normal. The results obtained are shown below.

Table V. *The effect on conductivity of additions of cane sugar and ethyl alcohol to normal solutions of citric acid.*

	$C \times 10^5$	Reduction of conductivity $\times 10^5$	a
Aqueous solution	553	—	—
Solution containing 2 % cane sugar ...	529	24	2·12
Solution containing 2·4 % ethyl alcohol...	497	56	4·21

The effect of alcohol on normal citric acid is thus nearly double that of cane sugar. Higher alcohols and esters appear to act in a similar manner and these may be of importance in reducing conductivity of the more acid fruit juices, but little is known as to the amount in which such substances are present in fruits.

There can be no doubt that the low conductivities which have been observed in fruit juices are due to two principal causes:

(1) The action of non-electrolytes.

(2) The mutual action of salts and acids in repressing dissociation.

Which factor will exert most influence must depend upon the nature and quantity of the acid present, the proportion of acid to salt and the concentration of non-electrolyte. Before it is possible to interpret the results of conductivity measurements in any given case further investigation is required of the influence of both salts and non-electrolytes upon the conductivity of organic acids, and also of non-electrolytes upon mixtures of these acids and their salts.

For the assumption that the influence of organic acids and salts on the conductivity is small and that the effect of non-electrolytes is usually negligible, Dixon and Atkins claim support from the results of certain experiments of Heald [1902] in which he found that the conductivity of certain plant juices was approximately equal to that of a solution of ash in a volume of water equal to that of the juice from which it was derived.

It must however be remembered that the relation of ash to sap is a very complex matter, and even were there no definite experimental evidence against the conclusion reached by Dixon and Atkins it would be hard to derive support for it from Heald's observations. The ashing of a sap involves a large number of changes which may affect conductivity. These include:

(1) The destruction of organic acids.

(2) The conversion of organic salts into carbonates some of which may be insoluble.

(3) The conversion of the metallic constituents of colloid substances (chlorophyll, proteins, pectins) into carbonates or oxides.

(4) The conversion of organic compounds of phosphorus and sulphur into phosphates and sulphates.

(5) The removal of non-electrolytes which reduce the conductivity.

How far these effects balance one another, must be largely fortuitous. In Heald's experiments the conductivity of the ash was usually found to be less than that of the sap, the difference varying from 2 per cent. to over 20 per cent. In the case of cucumber fruit—the only fruit investigated—the conductivity of the ash was only 60 per cent. that of the sap.

Dixon and Atkins' experiments on the effect of liquid air in rendering protoplasm permeable.

The foregoing considerations throw some light on an interesting series of experiments carried out by Dixon and Atkins [1913] on the differences in plant juices obtained by various methods. In these experiments a comparison was made between the juice from plant organs when pressed without previous treatment and the juice of the same organs when treated by exposure to liquid air before pressing. Determinations of freezing point and conductivity were made and on these were based conclusions as to the relative proportion of electrolytes and non-electrolytes present in each pair of samples. Dixon and Atkins found that in every case the freezing point of the juice from the treated organs was lower than that from the untreated, a clear indication that exposure to liquid air had reduced the resistance of the protoplasm to the passage out of the cell contents. They also found in many cases a corresponding but relatively less increase in conductivity which they interpret as showing that protoplasm in its untreated state is more permeable to electrolytes than to non-electrolytes.

In considering experiments of this nature it is desirable to form so far as possible some mental picture of the actual processes which take place. When living tissue is submitted to pressure it may be assumed that an exceedingly dilute solution of electrolytes will pass out at first, after which, as pressure is increased and a number of the cells are killed or burst, the greater part of their contents will escape. In the case of frozen tissues the cells of which are dead from the first, the escape of cell sap from the material will begin as soon as sufficient pressure is exerted. Dixon and Atkins assume that the difference in the value of the ratio $\frac{C}{\Delta}$ is evidence that a considerable quantity of electrolyte passes through the living protoplasm before it is killed by pressure. The interpretation of this ratio is, however, a matter of some difficulty and a further examination of Dixon and Atkins' work is necessary in order to draw definite conclusions from their experiments.

For this purpose a table compiled by Atkins [1916, p. 118] is quoted below which shows the effect of liquid air on the nature of the sap expressed from various plant organs.

		Δ	$C \times 10^5$	$\frac{C \times 10^3}{\Delta}$
(1)	*Hedera helix* leaves untreated ...	0·767°	403	5·2
	Same sample frozen	1·255°	605	4·8
(2)	*Ilex aquifolium* roots untreated	0·531°	563	10·6
	Same sample frozen	0·682°	629	9·2
(3)	*Ilex aquifolium* leaves untreated ...	0·651°	433	6·6
	Same sample frozen	1·130°	619	5·4
(4)	*Pyrus malus* fruit untreated	1·507°	171	1·1
	Same fruit frozen	1·919°	161	0·8
(5)	*Solanum tuberosum* tuber untreated ...	0·523°	555	11·0
	Same tuber frozen	0·588°	583	9·9
(6)	*Vitis vinifera* fruit untreated	2·567°	132	0·5
	Same sample frozen	3·185°	112	0·3
(7)	*Chamaerops humilis* leaf untreated ...	0·365°	298	8·1
	Same leaf frozen	1·529°	752	4·9
(8)	*Beta vulgaris* root untreated	1·473°	570	3·9
	Same root frozen	1·761°	555	3·2

It must be pointed out in the first instance that an increase in the total concentration of the constituents of the sap will not be accompanied by a proportional increase of conductivity, since the depression of the conductivity by non-electrolytes will also be proportionately increased. It is therefore necessary to consider in the first place whether Atkins was justified in the main conclusion which he drew from his experiments, viz. that the sap from the untreated tissue contained a higher proportion of electrolytes than that from tissue which had been previously frozen.

In order to obtain evidence upon this point the assumption is made here that the ratio of electrolytes to non-electrolytes in the two saps of each pair of samples from Atkins' table is the same, and that the relative decrease of conductivity in the sap from the frozen tissue is entirely due to the depression produced by the larger quantity of non-electrolyte it contains. On this hypothesis the amount of non-electrolyte can be calculated if a value be assumed for α. From Arrhenius's equation we have

$$\frac{C_2}{C_0} = \frac{100 - \alpha x}{100}$$

and

$$\frac{C_1}{C_0 p} = \frac{100 - \alpha p x}{100}$$

which gives

$$\frac{C_2 p}{C_1} = \frac{100 - \alpha x}{100 - \alpha p x}$$

where C_2, C_1 = the conductivities of the saps from the frozen and unfrozen organs.

C_0 = the conductivity of an aqueous solution corresponding to the more concentrated sap.

p = the ratio $\frac{\Delta_1}{\Delta_2}$, which by hypothesis is the ratio of electrolytes and non-electrolytes in the two saps.

x = the percentage concentration of non-electrolyte in the more concentrated sap.

Below are tabulated the values of x calculated from this equation from the values of Δ and $C \times 10^5$ observed by Dixon and Atkins and quoted above; the value assumed for a is 2.

Table VI.

	Δ_1	Δ_2	$C_1 \times 10^5$	$C_2 \times 10^5$	x %	x^1 %*
Hedera helix	0·767°	1·255°	403	605	9	12
Ilex aquifolium roots ...	0·531°	0·682°	563	629	20	6
leaves	0·651°	1·130°	433	619	17	10
Pyrus malus	1·507°	1·919°	171	161	31	18
Solanum tuberosum ...	0·523°	0·588°	555	583	19	6
Vitis vinifera	2·567°	3·185°	132	112	35	30
Chamaerops humilis ...	0·365°	1·529°	298	752	23	14
Beta vulgaris	1·473°	1·761°	570	555	29	17

* x^1 represents the percentage concentration of hexose the osmotic pressure of which corresponds to Δ_2. This is added for comparison. The corresponding quantity of cane sugar would of course be nearly double.

It must be remembered that equivalent conductivity will increase with dilution. In the case of acid saps this is very important, for the dissociation of most organic acids increases very rapidly as concentration diminishes. Increased dissociation may thus be largely responsible for the high value of x in such a case as that of *Pyrus malus*, or where the two samples of sap exhibit large differences of concentration.

Bearing these facts in mind a comparison of the values of x and x^1 does not appear to justify Dixon and Atkins' general conclusion that the proportion of electrolyte to non-electrolyte is higher in the saps from the untreated than in those from the treated tissues. In the majority of cases the differences observed in the ratio $\frac{C}{\Delta}$ can with great probability be accounted for by the fact that the electrolytes in the less concentrated saps are more highly dissociated and are subject to the action of a smaller quantity of non-electrolytes. In one or two cases, however, and notably those of *Ilex* and *Solanum*, it appears to be necessary to assume that there is an appreciable difference in the ratio of electrolyte to non-electrolyte in the two saps. This may be due to the expression of electrolyte from the untreated tissue before this becomes permeable to the other constituents of the cell sap as Dixon and Atkins assume, but it is clear that the same effect would be produced by the filtering out of non-electrolyte from the untreated tissue. If substance of high molecular weight were thus removed a considerable increase of conductivity might be produced while the accompanying change of osmotic pressure would be small and a small difference in concentration would accordingly account for the results obtained[1].

[1] It will be noticed that in three of the examples quoted from Atkins' table the conductivity of the sap from the frozen organ is less than that from the same organ untreated. It is suggested by Dixon and Atkins [1913, p. 430] that this is probably due to actual differences in the sap from different samples of the same organ, but that part of the effect may be due to viscosity. Differences of this magnitude would invalidate any conclusions that might be drawn from the experiments. It is however clear from the foregoing that it is entirely unnecessary to assume such differences.

One other point merits some further consideration. There is no doubt that the colloidal constituents of the sap tend to be filtered out in pressing and it is probable that the sap from treated tissues contains more of these substances than that from the untreated. It is therefore natural to suppose that this fact may provide an explanation of those cases in which the ratio of electrolytes to non-electrolytes varies in the two saps.

The case of *Solanum tuberosum* suggests the action of dextrin and it is natural to expect that pectin may exert a similar influence. There is, however, considerable doubt as to the part played by colloids in this respect, and Atkins has pointed out that the viscosity of solutions of this nature is not a measure of their influence upon conductivity. He instances the well-known case of gelatin gels and he describes an experiment in which a pectin solution was gelatinised and its viscosity therefore largely increased, while its conductivity remained unchanged. Atkins draws the conclusion that the effect of pectin upon conductivity is negligible and that this is true for the whole class of substances to which pectin belongs—this, however, is to go beyond the facts. Emulsoid colloids are two phase systems the two phases of which consist of solutions of the colloid in water and of water in the colloid. Conductivity appears to be conditioned by the composition of the more liquid phase only, but as this may contain a considerable quantity of the colloid the effect on conductivity may be far from negligible. All that Atkins' experiment appears to show is that the gelatinisation of pectin is a process which leaves the more liquid phase unchanged. It must be remembered, however, in regard to pectin that it has been observed to possess acid properties. Pectin and its salts may, therefore, have some conducting power in solution which will tend to counterbalance their effect on other electrolytes.

Until the action of colloidal substances has been further elucidated the complete interpretation of such observations as those of Dixon and Atkins cannot be attempted. It may however be stated with some assurance that their results indicate that in most cases the difference in the ratio of electrolyte to non-electrolyte in the saps from treated and untreated tissues must be very small, and that there is no evidence that any considerable quantity of electrolyte can be pressed out from the living cell. These conclusions are borne out by some experiments of André [1906, 1907] to which Atkins [1916, p. 119] has called attention. André determined the proportion of nitrogen and ash in samples of sap squeezed from various organs by successive pressures of 3, 12·5 and 25 kilos per square cm., and arrived at the conclusion that while the concentration of the substances present in the juice varied as the result of different pressures their relative proportions remained unchanged. The method was not susceptible of great accuracy. Small differences of concentration produced by filtration would scarcely be detected by this method especially if the substances filtered out contained neither mineral matter nor nitrogen. The experiments, however, lend some support to the view that any differences in the composition of the saps are mainly due to

the holding back of non-electrolytes by the untreated tissue in the course of filtration.

The foregoing analysis makes it clear that very great caution is needed in the interpretation of observations of the conductivity of plant saps. Decrease of conductivity may be occasioned not only by the addition of non-electrolyte but even in some cases by the addition of electrolyte, while changes of concentration often produce large changes in equivalent conductivity. It is hardly possible therefore to regard electrical conductivity as being in any sense a measure of content of electrolyte. Comparative measurements must nevertheless be of great value in the study of plant saps where methods are available by which the corresponding conductivity in aqueous solution can be ascertained.

It remains therefore to consider the possibility of introducing a correction by means of which conductivity measurements can be reduced to standard conditions.

Some indication of content of non-electrolyte can be obtained from freezing point determinations, but these are of little value for the present purpose, since change of conductivity is proportional to the actual content of non-electrolyte and not to its molecular concentration, so that *e.g.* a hydrolysis of cane sugar might take place which doubled the osmotic pressure, without producing an appreciable alteration of the conductivity. In saps which contain considerable quantities of sugar density determinations are of more value for this purpose, but the following appears to be the simplest method of determining the value of C_0 for a given sap.

By diluting with water to twice the volume the conductivity of the electrolyte in water is approximately halved and αx becomes $\frac{\alpha x}{2}$. If the conductivity of the diluted sap be then measured we have:

$$C_0(100 - \alpha x) = 100\, C_1$$

$$\frac{C_0}{2}\left(100 - \frac{\alpha x}{2}\right) = 100\, C_2$$

where C_1, C_2 are the measured conductivities.

From these equations the value of C_0 and αx should be obtained with fair accuracy in those cases in which the degree of dissociation varies very slightly with the dilution. When saps contain any considerable quantity of organic acids it is impossible to assume that the conductivity is halved by diluting to twice the volume. In these cases the matter is far more difficult especially as the degree of dissociation of the acid and consequently the effect of non-elctrolytes upon it will be largely determined by the amount of salts present in solution.

Further study of the action of both salts and non-electrolytes upon the conductivity of organic acids is necessary before helpful suggestions can be made in this connection.

SUMMARY.

1. Attention is drawn to the effect of non-electrolytes on the conductivity of electrolytes and a paper of Arrhenius is summarised in which the effect is investigated and its magnitude given for various cases.

2. An examination is made of the causes of the low values obtained in conductivity measurements in fruit juices containing considerable quantities of organic acids. It is pointed out that these low results must be ascribed to the action of non-electrolytes and of salts, but that further investigation of the mutual action of these two factors is necessary.

3. An attempt is made to estimate the influence of non-electrolytes and of the changes in the degree of dissociation produced by dilution in certain results obtained by Dixon and Atkins when comparing the saps obtained from frozen and unfrozen plant tissues. It is suggested that there is very little evidence for the marked differences which they assume to exist in the proportional composition of the two kinds of saps. It is further suggested that these experiments afford no evidence that the protoplasm of the cells of tissue under pressure is permeable to electrolyte to any considerable extent.

4. A formula is suggested by means of which in certain cases conductivity measurements may be reduced to standard conditions.

I desire to express my indebtedness to Professor V. H. Blackman for much help and criticism.

This work has been carried out in connection with an investigation of Cold Storage problems for the Food Investigation Board of the Department of Scientific and Industrial Research.

REFERENCES.

André (1906). *Compt. rend.*, **143**, 972.
—— (1907). *Compt. rend.*, **144**, 276.
Arrhenius (1892). *Zeitsch. physikal. Chem.*, **9**, 487.
Atkins (1916). *Some Recent Researches in Plant Physiology.*
Dixon and Atkins (1913). *Scientific Proc. Roy. Soc. Dublin*, 422, 434.
Haas (1917). *Bot. Gaz.*, **63**, 232.
Heald (1902). *Bot. Gaz.*, **34**.
Hempel (1917). *Compt. Rend. trav. Carlsberg*, **13**, 1.

XV. CONTRIBUTIONS TO THE STUDY OF THE VEGETABLE PROTEASES: I. INTRODUCTORY[1].

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(*Received March 22nd, 1919.*)

ALTHOUGH the work that has hitherto been done on the proteoclastic enzymes and their actions is considerable, the great bulk of it has been carried out in connection with those enzymes which occur in the animal body. Comparatively little has been done on the vegetable proteases[2]. This is possibly due to the fact that whereas the three types of animal proteases can be readily differentiated according to their origin, very different is the case with the

[1] This investigation was carried out at the South-Eastern Agricultural College, Wye, between 1912 and 1914. Publication was delayed owing to the author's absence on military service.

[2] Some confusion exists in the terminology of the proteoclastic enzymes. Euler [1912] calls them *carbamases* or *proteinases* and considers that animal pepsin is a *pepsinase* and trypsin a *tryptase*. Vines gives the name *peptase* to those enzymes which convert the higher more complex proteins into albumoses and peptones, and *ereptase* to those that split up albumoses and peptones into simple amino-acids.

The custom is rapidly becoming general of giving an enzyme a name derived from that of the substrate on which it acts by changing the final letter or syllable into *ase*. Thus lactase is the enzyme which hydrolyses lactose, urease that which decomposes urea into CO_2 and NH_3. According to this nomenclature the names used by Euler and by Vines are obviously incorrect for a tryptase would mean an enzyme which acts upon trypsin, an ereptase one that hydrolyses erepsin, and so on. It would be advisable in order to bring the terminology of the proteoclastic enzymes into line with that of other enzymes to apply the term *protease* to all those enzymes whether animal or vegetable in origin which will attack any of the proteins or their intermediate decomposition products. Those which attack the higher proteins only (*i.e.* those similar to pepsin) would be called *proteinases*, whereas those which split up albumoses, peptones, etc. but not the higher proteins (*i.e.* those similar to erepsin) would be *peptases*.

Animal trypsin is anomalous in some respects and has both proteinoclastic and peptoclastic properties. It cannot therefore be classed either as a proteinase or a peptase but only (generally) as a protease. It is not impossible though that trypsin may ultimately be found to be separable into a proteinase and a peptase [Vernon, 1904, 1; Vines, 1909]. Similar considerations apply to the vegetable ecto-proteases, *e.g.* of *Nepenthes*, which are considered to be mixtures of proteinases and peptases.

This terminology (which will be adhered to in the following paper) of course in no way conflicts with, or renders unnecessary, the classical individual names pepsin, trypsin and erepsin for the three particular enzymes so designated. Thus pepsin and erepsin are two particular enzymes belonging respectively to the classes proteinases and peptases.

vegetable proteases because a classification of these is not possible according to either the localisation of the enzymes or the nature of the media in which they act.

Three types of animal proteases are known:

1. *Pepsin*, which attacks so far as is known all true proteins. The products formed consist of albumoses and peptones, *i.e.* the hydrolysis is incomplete, lower polypeptides and amino-acids not as a rule appearing. Pepsin occurs in the gastric juice of all the vertebrates examined with the exception of certain fishes and it exerts its optimum activity in faintly acid solution, being quite inactive in an alkaline medium.

2. *Trypsin*, which is active in alkaline or neutral solution only, resolves proteins (as well as albumoses, peptones, etc.) into simple polypeptides and amino-acids. It is found in the pancreatic juice of all vertebrates, as well as in insects, protozoa, sponges, worms and molluscs.

3. *Erepsin* resembles trypsin in actively decomposing albumoses, peptones, protamines and polypeptides into simple amino-acids, but differs widely from it in its inability to decompose higher proteins such as fibrin and albumin. It occurs in the intestinal juice and intestinal mucous membrane and appears to be the most widely distributed of all the proteoclastic enzymes. According to Vernon [1904, 1, 2] the peptoclastic activity of trypsin is due to an ereptic enzyme (pancreato-erepsin) associated with it, so that it is conceivable that trypsin may be really separable into a pepsin and an erepsin (or rather a proteinoclastic and a peptoclastic part).

The vegetable proteases appear to be of two kinds which differ

(*a*) in mode of occurrence; and

(*b*) in the relations which exist between their digestive activity and the reaction, *i.e.* acidity or alkalinity, of the media in which they act.

One of these, and apparently the smaller class, consists of the extracellular or ecto-proteases which are found in the secretions of certain plants, *e.g.* in the antiseptic almost protein-free sap of the leaf pitchers of *Nepenthes*, in the leaf glands of *Dionaea* and *Drosera* and the leaf edge of *Pinguicula*, and also in bacteria especially those forms capable of liquefying gelatin. According to Vines [1897] the ecto-protease of *Nepenthes* is inactive in neutral and alkaline solutions, but is very active at the natural acidity of the pitcher liquid and also in the presence of free acid (up to 0·3 % HCl). This ecto-protease decomposes proteins not merely into albumoses and peptones but into amino-acids as well, and is considered by Vines to be a mixture of a proteinase and a peptase. On the other hand the ecto-protease of *Drosera* is active in alkaline and neutral as well as in acid media; it would appear therefore that the plant ecto-proteases are less rigid than the corresponding animal ones in respect to their activity in acid or alkaline media.

The second and larger class of vegetable proteases comprises the intracellular or endo-proteases most of our knowledge of which is due to Vines [1904, 1905, 1906, 1908, 1909, 1910]. They occur more especially in germi-

nating and ungerminated seeds and more abundantly in oil-bearing than in starch-containing seeds, and particularly in *Cannabis sativa*, *Sinapis*, *Ricinus* and *Linum*. They also occur in certain juicy fruits (*Ficus carica*) and leaves (*Agave*) and in many fungi such as yeasts. They belong to two groups (*a*) proteinases[1] and (*b*) peptases[1].

(*a*) The *proteinases* break down the true proteins such as fibrin and albumin, the decomposition however proceeding only as far as albumoses and peptones, behaving in this respect like animal pepsin. They are readily soluble in NaCl-solution but only slightly so in water or 50 % alcohol. Their extraction from seeds by means of water is therefore slow, as salts, etc., are extracted first, the enzymes dissolving in the solution so produced. Their digestive activity is greatest at the natural acidity of the plant extract. With pure aqueous extracts the activity is strong in neutral and even in slightly alkaline solution, but a slight addition of mineral acid (0·05 % HCl) or of rather more organic acid (0·30 % citric) arrests the action. Increase of alkalinity does the same. In this respect they differ from animal pepsin and the ecto-protease of *Nepenthes*, both of which show their optimum activity in the presence of free acids. In this connection it is worthy of note that Fernbach and Hubert [1900] regard the primary (acid) and secondary (basic) phosphates present in malt extracts as determining the course of protein-digestion, the former promoting, the latter retarding it.

The endo-proteinases very rarely occur alone in the organs of plants where proteins are decomposed but are usually (according to Vines always) accompanied by peptases which correspond with the erepsin of the animal body, differing from it only in their more extensive reaction range in the direction of acidity. As Vines points out, the fact that we never find proteinases apart from peptases must be correlated with the significance of the proteoclastic enzymes in the plant economy: proteinases alone would never be able to carry protein-hydrolysis far enough, as they hydrolyse only as far as albumoses and peptones.

(*b*) The *peptases* are readily soluble in water and in aqueous solutions of neutral salts. Their digestive activity is exclusively peptoclastic and is especially associated with an acid medium. The reaction range is not known as "pure" solutions of peptase have not been experimented with and the presence of other substances materially affects the action of acids and alkalies upon the enzyme.

Practically all the work done so far on the plant proteases has been confined to those that occur in seeds and in certain juicy fruits and insectivorous plants. Very little work has been done on the enzyme content of other parts of plants. The present investigation was undertaken with the object of determining whether proteoclastic enzymes exist in the ordinary foliage leaves and other parts of certain common farm crops and if so how far they

[1] Called by Vines peptases and ereptases respectively. Cf. note on p. 124.

resemble in their action the seed proteases. The crops experimented with, some fourteen in number, were grown on the college farm of the South-Eastern Agricultural College, Wye, in the spring and summer of 1912, some in the ordinary rotations and some specially for the purpose of this investigation. They belonged to the following species:

Cereals:	
Barley	*Hordeum sativum* (Pers.)
Oats	*Avena sativa* (L.)
Maize	*Zea mais*
Rye	*Secale cereale*
Leguminous plants:	
Red Clover	*Trifolium pratense* (L.)
White Clover	*Trifolium repens* (L.)
Lucerne	*Medicago sativa* (L.)
Sainfoin	*Onobrychis sativa* (L.)
Vetch	*Vicia sativa*
Field beans	*Vicia faba* (L.); *Vicia vulgaris* (Moench)
Peas (field var.)	*Pisum arvense* (L.)
„ (garden var.)	*Pisum sativum* (L.)
Other plants:	
Buckwheat	*Polygonum fagopyrum* (L.)
White mustard	*Brassica alba* (Vis.); *Sinapis alba* (L.)

They were examined at two stages of growth: in full flower and at a comparatively young stage when the plants were about 8—10 ins. in height. The cereals, however, were examined in the grass stage only. They were gathered, dried and ground as finely as possible in a steel mill and only the part that passed through a sieve of 80 meshes per mm. was used for the work. Owing to the wet summer of 1912 it was found impossible to air-dry them sufficiently to admit of more than very coarse grinding; so recourse was had to oven-drying. The partially dried chopped material was placed in shallow trays in a large iron oven heated by means of Bunsen burners placed underneath to a temperature not exceeding 45°—50°. In these circumstances it is practically certain that the injurious effects of the increased temperature on the enzymes present would be a minimum. It is well known that diastase can withstand higher temperatures than this even in aqueous solution and in common with all enzymes can withstand much higher temperatures when *in situ* than when in solution; *e.g.* in plant material diastase can be heated to 80° without being destroyed although its hydrolysing power is somewhat impaired. Miss White [1909] observed that heating dry oats to a temperature of 100° for 4½ hours was without effect on the enzymes present although they were destroyed by heating to 130° for one hour. The present author has observed that heating dry linseed cake in a steam oven to a temperature of 99° for 4 hours caused only a small reduction (less than 10 %) in the activity of the cyanogenetic

enzymes present. It is true that proteoclastic enzymes are more sensitive to heat than is diastase particularly in solution, but both substrate and decomposition products exert considerable protecting action (Bayliss, Vernon). Moreover, the optimum temperature of animal protease is given as 40°. The presence of such large quantities of adsorbent material as are present in green plants combined with the comparative dryness would tend to make the enzymes still more resistant to heat; so that in all probability no more damage would be done than in the case of simple air-drying under glass. At the same time oven-drying at this temperature facilitates grinding, enabling a much finer powder to be obtained.

In the case of such crops as lucerne and sainfoin which lignify rapidly very early in the flowering period, the main woody stems were rejected and only the leaves, flowers and smaller and younger twigs retained.

Owing to the bulkiness and comparatively small proteoclastic activity of the plant material and the dark colour of the extracts considerable difficulty was experienced in devising a method for estimating any enzymic activity there might be.

The method followed by Vines was first tried. This consists in detecting the presence of tryptophan (a frequent product of tryptic hydrolysis) by means of the violet colour produced by bromine water in the presence of a little HCl. The method requires considerable care and experience in its use before it can be used successfully even with "pure" trypsin and substrate, *i.e.* in colourless or nearly colourless solutions. In the present case the *differences* in activity exhibited by the various plant materials used were insufficient to be detected—let alone estimated—by this method. It was, therefore, abandoned as a quantitative measure but was used as a qualitative test for proteoclastic action since in every instance the controls gave no tryptophan reaction.

The method finally adopted was Sörensen's [1908]. By this method the extent of protein-hydrolysis can be determined from the number of free carboxyl groups formed. By adding excess of formaldehyde solution the free amino-groups of the amino-acids produced were neutralised forming methylene compounds; the carboxyl groups can then be estimated by titrating with $N/5$ $Ba(OH)_2$, using thymolphthalein as indicator. In this way the extent of protein-hydrolysis can be expressed by the number of cc. of $N/5$ $Ba(OH)_2$ used in the titration. Also on the assumption that each carboxyl-group formed during the hydrolysis corresponds with one amino-group, the amount of hydrolysis can be stated as mg. of nitrogen, this being obtained by multiplying the number of cc. of $N/5$ $Ba(OH)_2$ required by 2·8.

In using this method two solutions are required:

A. 0·50 g. of Grubler's thymolphthalein dissolved in 1000 cc. of 93 % alcohol;

B. 50 cc. of commercial formaldehyde (40 %) + 25 cc. of absolute alcohol + 10 cc. of thymolphthalein solution *A*. $N/5$ $Ba(OH)_2$ is run in until a faint

green or blue colour is obtained. This solution must be prepared fresh for each series of experiments.

In practice 10 g. of the plant material + 250 cc. of distilled water which had been raised to 40°[1] + a thin layer of toluene in a tightly corked flask served as a control.

10 g. of plant material + 5 g. of Witte's peptone + 250 cc. of water at 40°[1] + a thin layer of toluene; and 10 g. of plant material + 3 g. of legumin + 250 cc. of water at 40°[1] + a thin layer of toluene served as the experimental liquids. All three were contained in tightly corked flasks and were kept in an incubator at 37° for three days[2]. At the end of that time they were filtered as rapidly as possible, and the residues were thoroughly washed with cold water until the volume of the filtrate and washings from each was 400 cc. Preliminary experiments had shown that all amino-acids were washed out when the filtrate and washings occupied this volume. A tenth, *i.e.* 40 cc., of each filtrate was then transferred to a flask, decolorised as far as possible by shaking with alumina cream[3], filtered and the residue thoroughly washed. To the filtrate and washings from the control was added 15 cc. of solution *B* and *N*/5 $Ba(OH)_2$ run in until a distinct blue or green colour[4] was obtained. The other filtrates were then treated in the same way, *N*/5 $Ba(OH)_2$ being added to them until their tint matched that of the control solution. To the actual readings of $Ba(OH)_2$ used a correction had to be applied to allow for the amount of *N*/5 $Ba(OH)_2$ absorbed by the undecomposed protein, *i.e.* either legumin or peptone, left in the solution.

The results obtained for the action of 10 g. of plant material on 5 g. Witte's peptone during 3 days' incubation at 37° are given in Table I, A.

A consideration of the results given in Table I, A, indicates that a protease capable of splitting up Witte's peptone into simple amino-acids is present in all the plants examined. The peptoclastic activity varies with the nature of the plant but is an individual rather than a generic character. Thus, contrary to expectation, the leguminous plants examined did not on the whole possess greater peptoclastic power than the non-leguminous ones. The activity of the foliage of young peas (field variety) was found to be some $2\frac{1}{2}$ times that of young oat plants; but the leguminous plants other than peas were in no way

[1] This temperature was chosen in order that the temperature of the plant-water mixtures might be in the neighbourhood of 37° at the beginning of each experiment.

[2] This period was shown by preliminary experiments to be a suitable time for digestion. It was sufficiently long to allow a measurable amount of hydrolysis to occur but not long enough for the reaction to have reached its limit.

[3] The alumina cream was made by gradually adding excess of ammonia solution to saturated alum solution with constant shaking, allowing to stand for several hours, and then filtering off the precipitated alumina. It is very important that this should be washed until free from NH_3 and sulphate either of which would interfere with the titration. The alumina was then shaken up with water until a thin cream was obtained.

[4] Blue if the original solution was colourless, green if it was yellowish or brownish. Most of the extracts dealt with were deep brown in colour and after shaking with alumina cream were slightly yellow or brownish. This did not interfere with the titration provided all three filtrates were of the same tint.

superior to the non-leguminous ones, while white mustard (a non-leguminous plant) especially in the flowering stage was distinctly more active than any of the other plants examined except peas.

Table I.

Name of plant	A. *Witte's Peptone*[1]. N lib. in mg. = c.c. $N/5$ $Ba(OH)_2$ required for whole extract × 2·8	A. *Witte's Peptone*[1]. Percentage of peptone hydrolysed	B. *Legumin*[2]. N lib. in mg. = c.c. $N/5$ $Ba(OH)_2$ required for whole extract × 2·8	B. *Legumin*[2]. Percentage of legumin hydrolysed
Cereals (grass stage only):				
Barley	128·80	18·68	30·66	5·68
Oats	78·40	11·37	18·93	3·51
Maize	103·60	15·02	37·80	7·01
Rye	109·20	15·84	16·80	3·11
Leguminous plants:				
Red clover (young)	135·80	19·70	24·92	4·62
„ (old)	166·60	24·16	35·28	6·54
White clover (young) ...	134·40	19·49	22·68	4·20
„ (old)	165·20	23·96	30·24	5·61
Lucerne (young)	85·60	12·41	21·28	3·94
„ (old)	158·50	22·99	25·56	4·74
Sainfoin (young)	98·00	14·21	8·93	1·65
„ (old)	152·00	22·04	10·22	1·89
Vetch (young)	120·40	17·46	19·15	3·55
„ (old)	144·20	20·91	24·27	4·50
Beans (young)	81·20	11·78	10·22	1·89
„ (old)	102·20	14·82	15·34	2·84
Peas (f. var.) (young) ...	196·00	28·43	38·07	7·05
„ „ (old)	236·60	34·31	50·10	9·36
Peas (g. var.) (young) ...	140·00	20·30	39·20	7·27
„ „ (old)	177·80	25·79	47·88	8·88
Other plants:				
Buckwheat (young) ...	89·60	12·99	7·675	1·42
„ (old)	112·56	16·32	8·50	1·57
White mustard (young) ...	125·20	18·16	17·92	3·32
„ (old) ...	199·40	28·92	25·56	4·74

[1] Percentage of nitrogen in Witte's peptone = 13·79.
[2] Percentage of nitrogen in legumin = 17·97.

The activity varies with the stage of development of the plant and is in every case greater at the flowering period than in the earlier ones.

In some cases the tryptophan reaction was applied and gave positive results in some few instances with the plant-peptone extracts; with most of the plant extracts, however, as with the controls, no colour change occurred. It does not follow of course that no tryptophan was formed in these plant extracts either from the splitting up of the plant proteins themselves or from the added protein. In any case very little would be formed and this would be distributed throughout 400 cc. of liquid of which 10—20 cc. were taken

for the test. The absence of any tryptophan reaction is therefore quite explicable even though some slight formation of tryptophan had occurred.

In Table I, B, are given the results obtained for the action of 10 g. of plant material on 3 g. of legumin during 3 days' incubation at 37°.

The results given in Table I, B, are interesting in view of Vines' statement that out of a very large number of leaves examined by him only those of the lettuce and of *Phytolacca decandra* would digest blood fibrin or milk caseinogen. All other leaves gave either a doubtful or negative result. From this he concluded that the hydrolysis of albumoses and peptones by green plants is related to the fact that those proteins which occur naturally in the plant are such as are readily digested; whereas the more resistant ones similar to fibrin and caseinogen (which possibly do not occur in plant tissues, but only in the seeds where proteinases do undoubtedly occur) are not attacked.

The two results are, however, not altogether incompatible. Vines used fibrin in the form of small shreds in his experiments and relied on the appearance or, rather, the disappearance, of the fibrin after incubation as a test of hydrolysis. The legumin used by the present author was in the form of a very fine powder; the surface exposed to any proteoclastic action there might be would be much larger than in the former case and digestion therefore more rapid. The method employed for detecting the hydrolysis was also a more delicate one and even under these favourable conditions the hydrolysis was slight as compared with that of the peptone. It is doubtful whether such a small hydrolysis would produce any *visible* effect on small shreds of fibrin suspended in the extracts.

That the activity indicated is enzymic in nature and not merely the result of imperfections in the conditions of experiment seems to be shown by the fact that, as with peptone, the activity is invariably greater at the flowering stage than at the earlier one, and the greater activity of peas is again evident when legumin is employed as the substrate.

Having established the presence of proteinoclastic and peptoclastic enzymes in all the green plants examined it was next decided to examine one or two plants in detail in order to find out how the different parts of the plants varied as regards enzyme content. For this purpose an examination was made of beans, peas (field variety) and buckwheat. In this portion of the work the different parts of the plant were gathered separately and dried—first by air-drying and then *in vacuo* over sulphuric acid.

To test the effect of germination the seeds were germinated in moist sand kept in a warm place, were dried as rapidly as possible in an air-oven at 40—45°, coarsely ground and finally dried *in vacuo* over sulphuric acid. The enzymic activity was determined by Sörensen's method as described above. The results are given in Table II, A and B.

An examination of the tables shows that all parts of the plant are active and at all stages of growth. The activity of the leaves and seeds presents features of particular interest. Both the proteinoclastic and peptoclastic

activity of the leaves increases with increasing maturity right up to the time of harvesting and does not fall off, as was at first anticipated, after the flowering stage when translocation of foodstuffs from the leaves to other parts of the plant is taking place and during the dying off of the leaves. In this connection it is interesting that increasing activity with increasing maturity of the foliage right up to the time at which the leaves begin to die off has been shown to hold also for the cyanogenetic enzymes present in flax foliage. Thus the foliage of flax in full flower has been found to be rich in both cyanogenetic enzyme and cyanophoric glucoside; whereas flax leaves which have turned brown at the edges are still as rich in enzyme some time after the corresponding glucoside has completely disappeared.

Table II.

Part of plant	A. *Witte's Peptone.* N lib. in mg. =c.c. $N/5$ $Ba(OH)_2$ required for whole extract ×2·8	A. *Witte's Peptone.* Percentage of peptone hydrolysed	B. *Legumin.* N lib. in mg. =c.c. $N/5$ $Ba(OH)_2$ required for whole extract ×2·8	B. *Legumin.* Percentage of legumin hydrolysed
Beans:				
Leaves (plant 3–4 in. high)	81·20	11·77	10·22	1·73
,, (full flower) ...	102·20	14·82	15·34	2·84
,, (at harvest) ...	159·80	23·71	17·92	3·32
Flowers	153·40	22·25	19·18	3·56
Seeds (ungerminated) ...	154·65	22·43	14·06	2·61
,, (just germinated) ...	188·20	27·29	12·77	2·37
, (germinated) ...	230·10	33·37	14·06	2·61
Pods	139·45	20·22	10·00	1·74
Stems	93·50	13·56	14·06	2·61
Peas (field var.):				
Leaves (plant 6–8 in. high)	196·00	28·42	38·08	7·06
,, (full flower) ...	236·60	34·32	50·96	9·45
,, (at harvest) ...	276·10	40·04	55·44	10·38
Flowers[1]	171·40	24·85	25·56	4·74
Seeds (ungerminated) ...	120·40	17·46	24·08	4·47
,, (germinated) ...	244·10	35·41	47·04	8·80
Pods	145·00	21·03	12·60	2·34
Stems	83·44	12·10	13·44	2·49
Buckwheat:				
Leaves (v. young)... ...	89·60	12·99	7·67	1·42
,, (full flower) ...	112·55	16·32	8·95	1·66
,, (at harvest) ...	222·40	32·25	10·22	1·90
Flowers	122·60	17·79	35·78	6·64
Seeds (ungerminated) (6 dys.)	103·40	15·00	5·18	0·96
,, (just germinated) ...	114·80	16·65	12·77	2·37
,, (germinated) ...	177·90	25·21	—	—

[1] This figure is only approximate as the pigment present interfered with the titration and made the end-point indeterminate.

The effect of germination on the enzyme content is also of considerable interest. It would appear from the results obtained that ungerminated seeds,

like all other parts of the plant, are active towards Witte's peptone and slightly so towards legumin. When germinated so that the young shoot is just showing itself a distinct increase in peptoclastic activity occurs; when the germination has proceeded until the shoots are one to two inches in length both the proteinoclastic and peptoclastic activity become much greater. This is shown best in the case of buckwheat and falls in line with Vines' observations that the activity is not at once evident but is developed during the germination process[1].

The above investigation also throws a little light on another interesting point. Vines [1903] first pointed out the possibility of the proteinoclastic and peptoclastic enzymes of green fodder crops being of use to the animal during the process of digestion. He pointed out that in the case of herbivorous animals and especially the ruminants the vegetable food that has been eaten is probably placed under conditions that are altogether favourable to the action of the proteases which it contains, so that there is reason to believe that digestion in these animals is, in no small degree, a process of autolysis, the food providing at once the nutriment and the means of digesting it. It is by no means impossible too that the enzymes present may even assist in the digestion of any additional protein that may be fed along with the greenstuff. The results obtained above indicate that the peptoclastic activity of green fodder plants is sufficiently great to be of assistance to the animal in the digestion of the simpler, more easily digested, proteins, but that the proteinoclastic activity is much too slight to be a serious factor in the digestion of the higher proteins that may be present in, or fed along with, the green food.

Whether the conditions operative in the animal's stomach are such as admit of auto-digestion of proteins taking place is another matter and cannot be determined until we know the quantitative relationship that exists between the peptoclastic activity of plant proteases and the reaction of the medium in which they act. Attempts were made to determine this but with little or no success. Various aqueous extracts of the plant material were obtained and the action of acids and alkalies upon their peptoclastic activity was investigated. In the case of the alkalies more or less bulky precipitates were formed in all cases during the incubation and no consistent results could be obtained. This is not very surprising when it is remembered that plant extracts are very slightly acid in reaction. On changing the reaction of the medium the internal equilibrium is disturbed and the colloidal substances present are partially precipitated carrying some enzyme down with them.

The whole extract is an extraordinarily complex colloidal system and even

[1] The sudden increase in peptoclastic activity that occurs in seeds during germination finds an interesting parallel in the case of the lipase of linseed. While a lipase is frequently found in many oil-bearing seeds—being especially abundant in species of *Ricinus*—its presence had not until recently been detected in linseed although many attempts have been made to find it. The present author, in conjunction with Dr J. V. Eyre, has succeeded in showing the presence of a lipase in *germinated* linseed and has measured its activity. It would appear to be developed during the germination process.

if no disturbing factors were introduced and it were possible to determine accurately the effects of acids and alkalies on the enzymic activity the results obtained would not necessarily correspond to those obtained when using "pure" enzyme preparations. All that can be deduced from these latter experiments is that the peptoclastic activity of plant extracts is not destroyed by the addition of small quantities (*e.g.* up to 0·05 %) of either acids or alkalies. But the direct action of acids or alkalies cannot be determined until the enzymes can be extracted in a less impure condition. This is being attempted, and it is hoped that a systematic study of the vegetable peptases may form the subject of a further communication.

REFERENCES.

Euler (1912). *General Chemistry of the Enzymes.*
Fernbach and Hubert (1900). *Compt. rend.*, **131**, 293.
Sörensen (1908). *Biochem. Zeitsch.*, **7**, 45.
Vernon (1904, 1). *J. Physiol.*, **31**, 346.
—— (1904, 2). *J. Physiol.*, **32**, 33.
Vines (1897). *Ann. Botany*, **11**, 563.
—— (1903). *Proc. Linn. Soc.* (London), 18 and 42.
—— (1904, 1). *Proc. Linn. Soc.* (London), 59.
—— (1904, 2). *Ann. Botany*, **18**, 289.
—— (1905, 1) *Ann. Botany*, **19**, 149.
—— (1905, 2). *Ann. Botany*, **19**, 171.
—— (1906). *Ann. Botany*, **20**, 113.
—— (1908). *Ann. Botany*, **22**, 103.
—— (1909). *Ann. Botany*, **23**, 1.
—— (1910). *Ann. Botany*, **24**, 213
White (1909). *Proc. Roy. Soc.*, B. **81**. 417

XVI. ON THE ESTIMATION OF SUGAR IN BLOOD.

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(*Received April 15th, 1919.*)

In spite of the large numbers of determinations of blood sugar that have been carried out by various means in the past few years there is still need for a reliable and simple method.

Much work has recently been done with the picric acid procedure in which the depth of colour produced is estimated by means of the colorimeter. It is, however, a drawback to this method that a colorimeter is not always available especially for clinical work. Further, it is doubtful whether this method is at all applicable in the case of whole blood on account of the creatinine (and probably other interfering substances present) which, under the conditions of the estimation, give a colour similar to that produced by glucose in the presence of picric acid. De Wesselow points out [1919] in the following paper that these interfering substances are always active and in the case of human blood may actually influence the picric acid method to such an extent that the result obtained may be as much as 50 % too high. In plasma also the method appears to yield results on the high side, but here the figures obtained are probably accurate enough for practical purposes.

Bang in 1913 introduced a micro-method for blood sugar determination, but the difficulty of completely separating the protein and the ease with which oxidation of the cuprous oxide took place before and during titration interfered very materially with its practical utility.

A new application in which the tendency to oxidation of cuprous oxide during titration was completely overcome was introduced by the writer a few years ago. This method was first used in 1914 and was communicated to the Biochemical Society in autumn 1915. A few months after this communication was given, a method based on the same principle was published independently by Scales [1915] while my paper was actually in the press [MacLean, 1916]. This method gives excellent results and the fact that Bang [1918] adapted this principle to his own method speaks for itself. The application of the method for the estimation of blood sugar was described by me in the above noted publication [MacLean, 1916]. An extended use of the procedure, however, has resulted in various improvements and considerable simplification, and it is felt that the modified method as described in

the present paper not only gives very reliable results, but is so simple in its application that it can be carried out by students and can easily be utilised for clinical purposes.

Principle of the Method.

In estimating the sugar in blood it is first necessary to separate the protein very completely. For this purpose various procedures were tried, but the use of dialysed iron in conjunction with heat coagulation gave the best results. In the new method the blood is heated in an acid saline solution and the greater part of the protein separated by coagulation. Any remaining traces of protein are removed by the addition of a small amount of dialysed iron. After cooling, the mixture is filtered and a perfectly clear protein-free filtrate obtained. The sugar in an aliquot part of this filtrate is estimated by boiling the liquid with an alkaline copper solution containing potassium iodate and iodide. After boiling, the solution containing the reduced cuprous oxide in suspension is cooled and treated with a slight excess of hydrochloric acid. This interacts with the potassium iodate and iodide liberating iodine equivalent to the amount of potassium iodate in the solution. At the same time the cuprous oxide is dissolved and cuprous chloride formed. A solution of the latter is very unstable and liable to oxidation, but this is prevented by the fact that free iodine is present with which it immediately reacts. Since the cuprous oxide present in the mixture at an earlier stage is not in solution but in suspension, no oxidation takes place during the preliminary cooling. During the boiling, any air present in the solution is driven off before the sugar has time to reduce the copper to any appreciable extent; also the flask is full of steam during this time so that no oxidation from air oxygen need be feared.

The amount of sugar present in the solution is calculated by ascertaining the amount of iodine used up as fully described later.

The only point in the method that requires special care is the regulation of the flame used to boil the sugar and alkaline copper solution. The tables given in this paper are worked out for a flame which brings the amounts of solution specified later to the boil in as nearly as possible one minute forty seconds. In this time the *boiling should be fully established;* often some bubbles come off 10 seconds or so before the boiling becomes brisk, but it is usually quite easy to ascertain the point of complete boiling to within a few seconds. For clinical purposes a rough preliminary standardisation of the flame is all that is required, but for very accurate work the use of a small manometer is necessary. When care is taken to follow the directions given the results obtained vary only within the limits of the ordinary experimental error of manipulation which of course is a personal factor. With pure solutions of glucose successive identical readings are obtained with monotonous regularity if the conditions are carefully observed. With blood also duplicate experiments yield practically identical figures (see below).

One detail in the method requires some comment. When hydrochloric acid is added to the boiled mixture of alkaline copper solution and cuprous oxide the acid reacts with the carbonates present and effervescence takes place. Since free iodine is present at this stage it might be expected that this effervescence would result in driving off some of the iodine. That this may be the case to some infinitesimal extent is probable, but I have satisfied myself that when ordinary care is taken not to agitate the mixture too vigorously until the effervescence passes over no appreciable amount of iodine capable of estimation is lost. This probably depends on the extreme dilution of the iodine solution.

For blood sugar determinations the method is applicable to 1 cc. or smaller amounts and descriptions of the procedure for estimating sugar in 1 cc. and in 0·2 cc. of blood respectively are given here. The use of such a small amount as 0·2 cc. blood has various advantages, since this amount can be easily obtained from the finger. For 1 cc. it is necessary to insert a needle into a vein of the arm, and if observations are required at frequent intervals this procedure becomes difficult for obvious reasons. On the other hand, quantities of 0·2 cc. blood can be obtained from the ear or finger as often as may be required.

In all blood sugar estimations it is important to remember that glycolysis is often very active especially for a short time after the blood is drawn; for this reason the specimen should be immediately measured and added to the coagulating mixture described and, if convenient, heated at once. By this means glycolysis is completely prevented. After standing for two hours at room temperature the amount of sugar present in the specimen may be less than half its original sugar content. Since the method of obtaining 0·2 cc. blood described later allows of almost immediate mixing of the blood and coagulating fluid, the use of this small amount has an additional advantage.

Detailed instructions for the preparation of the various solutions required are given below. An objection urged against the method was the difficulty of preparing an accurate $N/10$ thiosulphate solution, but this difficulty no longer exists since standardisation against potassium bichromate is now used.

Solutions required for 1 cc. Method and their preparation[1].

The solutions required have the following composition and are prepared according to the directions given.

A. *Solutions for removing protein from blood.*

No. 1. *An acidified sodium sulphate solution.*

Sodium sulphate (pure)	150 g.
Glacial acetic acid	3 cc.
Distilled water to	1000 cc.

[1] All these solutions may be obtained ready for use from the British Drug Houses, Graham Street, London.

Solution should be filtered through a starch-free filter paper (Whatman No. 1).

No. 2. Dialysed iron solution.

This solution should be about B.P. strength and well dialysed. The majority of the samples on the market are practically not dialysed at all and contain very large amounts of chlorides. A suitable preparation is stocked by the British drug houses under the term "Dialysed iron B.D.H."

B. *Solutions for estimating sugar in filtrate.*

No. 3. Alkaline copper iodide solution.

Potassium bicarbonate	20	g.
Potassium carbonate (anhydrous)	15	,,
Copper sulphate crystals	0·7	,,
Potassium iodate	0·11	,,
Potassium iodide	1	,,
Distilled water to	100	cc.

In making up the solution 20 g. of potassium bicarbonate are dissolved by gentle heating in 60 to 70 cc. of distilled water. The heating must be very carefully done and the temperature of the mixture should not exceed 37°. The potassium carbonate is then added. The copper sulphate is dissolved in a separate beaker in a few cc. of water and added to the mixture of carbonates without waiting for the potassium carbonate to dissolve completely. After the resulting effervescence has passed over, the complete solution of any remaining carbonate is accomplished by heating. The iodate and iodide are next added, the solution thoroughly shaken and made up to 100 cc. After filtering through a starch-free paper (No. 1 Whatman paper) it is ready for use. The solution keeps indefinitely. The copper sulphate and potassium iodate must be weighed with great care. The other ingredients need not be so carefully measured.

This solution must be standardised as regards its iodine content by taking exactly 3 cc. of the mixture and adding this to 10 cc. of the 15 % sodium sulphate acetic acid solution used for deproteinising the blood. To this mixture 10 cc. of 20 % hydrochloric acid are added. The flask is very gently agitated until effervescence passes over and then more strongly agitated for 1 minute. A solution of $N/100$ sodium thiosulphate is run in until the yellow colour of the iodine has almost completely disappeared. Two drops of starch solution are then added and the titration proceeded with until the blue colour disappears. The number of cc. of thiosulphate used up gives the equivalent of iodine liberated from the mixture and should be recorded on the bottle. For 3 cc. copper iodide solution about 8·8 cc. of $N/100$ thiosulphate are required. The exact figure obtained depends to some extent on the purity of the samples of iodate and iodide used, but variations due to this should be very slight. The samples used in my work give exactly 8·82 cc. thiosulphate = 3 cc. copper solution.

No. 4. (*N*/10 *thiosulphate solution.*)

Though sodium thiosulphate crystals can be obtained in a state of great purity it is generally stated that the salt cannot be weighed directly for the purpose of making a standard solution owing to the possibility of water or air being held within the crystals. This difficulty may be avoided by treating the finely ground crystals with pure alcohol and then with ether and finally drying at room temperature. This manipulation, however, is tedious and there is always some slight risk that the crystals may not have been dried in air for a sufficiently long time to give an ultimate product containing the correct amount of water of crystallisation. The preparation of an accurate iodine solution for standardisation by weighing the iodine is also a tedious procedure. To avoid all these difficulties, the sodium thiosulphate solution used for this method is prepared by standardising against an *N*/10 solution of potassium bichromate. In preparing the *N*/10 thiosulphate a solution of *N*/10 potassium bichromate is first prepared by dissolving 4·913 g. of the pure dried salt in distilled water and making up the volume to 1000 cc.

The distilled water used for the thiosulphate solution should be boiled immediately before use to get rid of traces of CO_2. About 26 g. of pure sodium thiosulphate crystals are dissolved in about 1000 cc. of the cooled, boiled out water. To estimate the strength of this solution 20 cc. of *N*/10 bichromate are mixed in a flask with 10 cc. of 10 % potassium iodide and 5 cc. of strong hydrochloric acid. On shaking slightly the reaction is complete and exactly 0·254 g. iodine is liberated. The thiosulphate solution is run in from a burette until the brownish yellow colour has almost disappeared. One or two drops of a starch solution are then added and the titration continued until the liquid passes from a dirty dark yellow to a bright green colour. The end point is very sharp and cannot be mistaken. The number of cc. of thiosulphate used up indicates the strength of the thiosulphate solution. Since, however, all samples of potassium iodide contain traces of iodates, the iodide solution alone without bichromate will yield a very small amount of iodine in the presence of acid. This must be allowed for by making a blank experiment with 10 cc. potassium iodide and 5 cc. hydrochloric acid and titrating with thiosulphate as before. Not more than 0·1 to 0·2 cc. of thiosulphate should be used up. This must be subtracted from the result obtained with the bichromate. If say 18·7 cc. thiosulphate were required and the potassium iodide gave a blank of 0·2 cc., then the amount of thiosulphate solution required to combine with 0·2 g. of iodine would be 18·7 − 0·2 = 18·5 cc. This means that to obtain an *N*/10 solution each 18·5 cc. must be diluted to 20 cc., or that a litre will contain 925 cc. of the solution. To complete the procedure 75 cc. of boiled-out water are introduced into a 1000 cc. measuring flask from a burette and the volume made up to 1000 cc. with the thiosulphate solution. This gives an accurate *N*/10 solution which may be tested against 20 cc. of bichromate as

described. The solution will keep for at least several months if preserved in a well stoppered coloured bottle in the dark.

To prepare an $N/100$ solution 20 cc. of the $N/10$ solution are made up to 200 cc. in a measuring flask with distilled water. This weak solution is liable to deteriorate and should be made up fresh every few days. It is important to remember that burettes in which thiosulphate solutions are kept must be cleaned at frequent intervals with a solution of sulphuric acid and bichromate.

No. 5. *Starch solution.*

This is prepared by dissolving about 1 g. of "soluble" starch in 100 cc. distilled water. The solution should be freshly made up though an old solution can be used with success.

No. 6. 20 % *hydrochloric acid solution.*

Prepared by diluting 20 cc. of concentrated hydrochloric (B.P. Sp. gr. 1·16) with distilled water to 100 cc.

STANDARDISING OF HEATING.

This can be very easily accomplished by interposing a small manometer between the gas tap and the burner. A suitable manometer has already been described by Cole [1914], but since the question of temperature is so important, a rough sketch of the most suitable heating and regulating arrangement is given here (Fig. 1). If a suitable manometer is not to hand it is very easy to extemporise one, for all that is required is a T-piece with two pieces of bent glass. These can be connected by means of rubber tubing and are quite as effective as if the whole were in one part. A piece of rubber tubing passing through a screw clip connects the one end of the T-piece with the gas supply, while the other end is connected with the bunsen burner. The burner should have a small chimney to protect the flame. From $\frac{1}{4}$ to $\frac{1}{2}$ inch above this, a ring with ordinary wire gauze is supported, on which the flask is boiled. The gauze must be separated by an interval from the chimney, otherwise the gas will not burn properly. When a suitable flame is determined the position at which the cover of the air inlet cuts the side of the hole in the inner side of the bunsen is marked with a file, as indicated in the diagram, so that the same amount of air can always be admitted by adjusting to this mark. Before proceeding to find a suitable strength of flame, some coloured fluid is put in to the manometer through the open end by means of a capillary pipette; the upper surface of this fluid in both tubes is then indicated by a line on a white card placed behind the manometer or by marks on the two tubes of the manometer itself. When this is done the gas is turned full on, the screw closed down somewhat, and a trial made by placing a conical Erlenmeyer flask containing 23 cc. of sodium sulphate acetic acid solution over the flame. The fluid should be first cooled (or warmed) to 20° C. After a few trials, a flame is easily found which will bring the solution to vigorous boiling in about

1 minute 40 seconds. This is then controlled by taking 20 cc. of above sodium sulphate solution to which are added 3 cc. of the alkaline copper iodine solution and testing as before. In each case, of course, the adjustment of the flame is brought about by loosening or tightening the screw clip. When a strength of flame is found that brings the mixture to *distinct boiling* in about 1 minute 40 seconds to 1 minute 45 seconds, the position of the fluid in both limbs of the manometer is marked (see Fig. 1) either by ink on the glass or on a white card behind, or the difference of level read off and noted. The regulating mechanism is now complete, and whenever it is required at a future time all that is necessary is to adjust the gas pressure by means of the screw until the upper surfaces of the coloured liquid stand at the marks indicated. After some time it will be necessary to add more fluid to compensate for slight losses due to evaporation.

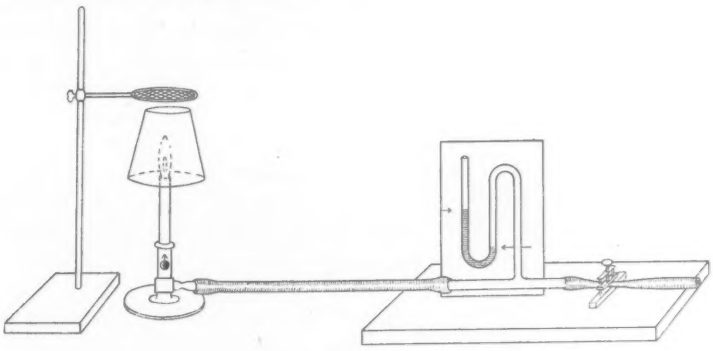

Fig. 1.

The gas pressure in this laboratory varies considerably on some days, while on others it remains practically constant for hours. During careful experiments the manometer must be watched and if a change of pressure occurs the necessary adjustment must be made by means of the screw clip.

I. Details of Method when 1 cc. of Blood is used.

Coagulation of protein.

Exactly 26 cc. of acid sodium sulphate solution are run into a small Erlenmeyer flask from a burette. The flask is fitted with a rubber stopper through which passes a glass tube terminating in a capillary point. Blood is drawn from a vein into a test tube containing a small amount of finely ground potassium oxalate and the tube inverted a few times. One cubic centimetre of the blood is measured in a special pipette made by Hawksley "to contain" 1 cc. The blood is allowed to flow out on to the surface of the fluid in the flask. Any blood adhering to the pipette is then washed out by alternately

sucking up and blowing out several times some of the sodium sulphate solution. If the blood is delivered carefully at one side of the flask to begin with, it floats more or less on the surface, leaving the fluid in the other parts of the flask almost clear. If care is taken, the pipette can be washed out with practically blood-free fluid, so that every trace of blood can be delivered. The special stopper with glass tube is now placed firmly in the flask and the mixture heated over a free flame until a bubble or two just appear indicating that the fluid is approaching the boiling point. At this point the flask should be removed. It is then shaken by a circular motion and allowed to stand for one or two minutes. The stopper is then carefully loosened and withdrawn sufficiently to allow the entry of the point of a pipette containing 3 cc. dialysed iron. This iron is added, the flask being shaken gently all the time. It is then shaken two or three times taking care that any drops of fluid in the neighbourhood of the stopper or neck of the flask are washed into the general contents. The flask is then cooled under the tap and the mixture is ready for filtration.

Practically no fluid is lost by coagulating the mixture over the free flame since the small capillary tube does not permit of the escape of much steam while the expanded air makes its exit. Indeed the upper part of the flask remains sufficiently cool to condense steam until the liquid is almost boiling. Even if a little steam does pass into the tube, it condenses there and is sucked back into the flask when air is drawn in on cooling. The large volume of fluid used also minimises any effect of possible loss of traces of moisture by this method.

Filtration.

The contents of the flask are now filtered through a 9 cm. Whatman No. 1 filter paper (W. & R. Balston). This paper is starch-free and I have always found it most suitable for this purpose. Filtration is allowed to proceed until the whole of the liquid has passed through the filter paper; this requires about 6 or 7 minutes. From 23 to 24 cc. of filtrate are obtained. Of this 20 cc. are taken if the blood is from a normal person and likely to contain an average amount of sugar. In the case of diabetics, or if glycosuria is present, 10 cc. filtrate is sufficient. In the latter case the total is made up to 20 cc. by the addition of 10 cc. acid sodium sulphate mixture. These amounts are measured into a small conical Erlenmeyer flask of hard heat-resisting glass. To this exactly 3 cc. of the copper iodide solution is added and the mixture heated.

Heating blood filtrate containing alkaline copper solution.

Over a flame suitably adjusted as already described to bring the 23 cc. of liquid to boiling in about 1 minute 40 seconds the solution is boiled for exactly 6 minutes from the point at which distinct boiling begins. The flask is then removed and either cooled under the tap, or plunged up to its neck into a large volume of cold water in the sink and kept there for about 1 minute. Thorough cooling is essential and must always be carefully carried out.

Treatment of boiled solution.

To the cooled solution are now added carefully with a pipette 10 cc. of 20 % hydrochloric acid and the flask *very gently agitated* at intervals until the effervescence passes over. From this point it is left to stand for about 1 minute or a little more, being shaken with a strong circular motion every few seconds. At the end of this time $N/100$ thiosulphate is run in from a burette and the solution titrated until the yellow colour almost disappears. Two drops of 1 % starch solution are now added and the titration completed. As already stated it is perhaps best not to add the starch at the beginning of titration for there is no doubt that the end point is more distinct if done as indicated here. When carried out in this way, the end point is exceedingly sharp and it is quite impossible for anyone to be more than one drop out. After the titration is finished there is a tendency for the blue colour to return as is usually the case in such solutions, but since this does not happen for several minutes it in no way interferes with the titration.

Calculation of result.

Suppose the blood filtrate as treated above requires 6·69 cc. $N/100$ thiosulphate and 3 cc. of the copper solution alone requires 8·85 cc., then the difference between 8·85 and 6·69 = 2·16 cc. must be due to the iodine absorbed by the cuprous chloride formed. Now from Table I it is seen that 2·16 cc. $N/100$ thiosulphate = 0·6 mg. sugar. Since the amount of filtrate taken corresponds to $\frac{2}{3}$ of 1 cc. of blood, it is obvious that 1 cc. blood contains 0·9 mg. sugar or that 100 cc. of blood contains 0·09 g. of sugar.

Table I.

(For estimation of sugar in 1 cc. blood.)

Showing equivalent of glucose to $N/100$ thiosulphate solution.

Glucose mg.	$N/100$ thiosulphate c.c.	Glucose mg.	$N/100$ thiosulphate c.c.
0·2	0·55	1·2	4·36
0·3	0·95	1·3	4·71
0·4	1·34	1·4	5·07
0·5	1·76	1·5	5·42
0·6	2·16	1·6	5·78
0·7	2·52	1·7	6·13
0·8	2·88	1·8	6·49
0·9	3·27	1·9	6·84
1·0	3·65	2·0	7·20
1·1	4·00		

That the method gives very consistent results is obvious from the following figures obtained on duplicates in six different specimens of blood. Three of

these experiments were carried out on fresh blood, while the others were done on specimens which had stood in the laboratory from 3 to 24 hours.

Fresh bloods			After standing for some hours		
No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
0·096 %	0·102 %	0·089 %	0·059 %	0·048 %	0·030 %
0·098 %	0·108 %	0·086 %	0·066 %	0·046 %	0·030 %

II. Method for Estimating Sugar in 0·2 cc. Blood.

The practical advantages of using a small amount of blood have already been discussed and since the manipulation here is quite as simple as is the case when 1 cc. of blood is used, this "micro-method" should prove very useful from the clinical standpoint. This method was partly worked out in 1915, but at the time I was unable to complete it. If the conditions given here are carefully observed, the results obtained are of the same order of accuracy as those in the above method and in spite of the fact that such a small quantity of blood is used no delicate manipulation is required. For this method the following solutions are required. Some of them are similar to those used for the 1 cc. procedure while others are considerably modified.

A. *Solutions for coagulating blood.*

No. 1.

Sodium sulphate	150 g.
Glacial acetic acid[1]	1 cc.
Distilled water to	1000 cc.

No. 2.

Dialysed iron solution "Dialysed iron B.D.H."

B. *Solutions for estimating sugar in filtrate.*

No. 3.

Potassium bicarbonate	12 g.
Potassium carbonate (anhyd.)	8 ,,
Copper sulphate crystals	0·35 ,,
Potassium iodate	0·05 ,,
Potassium iodide	0·5 ,,
Distilled water to	100 cc.

This is prepared as described under the 1 cc. blood method. It is standardised by taking 2 cc. in 10 cc. acid sodium sulphate solution and adding 2 cc. 75 % HCl. One minute after effervescence is completed and after the mixture has been thoroughly shaken it is titrated as already described with $N/400$ thiosulphate. 2 cc. of this copper solution should require about 11·05 cc. $N/400$ thiosulphate.

[1] The acetic acid should not be added until the solution is required for use.

No. 4.

An $N/400$ sodium thiosulphate solution is prepared from a $N/10$ solution by adding 5 cc. of the latter to some distilled water in a flask and making up to 200 cc.

No. 5.

A solution of "soluble starch" of about 1 % strength.

No. 6.

75 % HCl solution made by diluting 75 cc. concentrated HCl (B.P. Sp. gr. 1·16) to 100 cc. with distilled water.

Details of Method using 0·2 cc. Blood.

The blood is obtained directly from a finger or from the ear. To measure and collect the blood a special pipette[1] is used (Fig. 2), which is shaped so that blood flows in from a slight puncture. If the finger is used it is rubbed with a little ether and thoroughly dried with a cloth. A piece of rubber tubing is then wound round the upper part to cause congestion towards the point and a prick is made with a needle on the back of the finger a little above the root of the nail. The point of the pipette is placed in contact with the side of the drop, when the blood immediately flows in. The pipette is then held horizontally or with the distal end somewhat inclined downwards, so that the passage of the blood into the pipette is helped by gravity. When the blood reaches the mark the pipette is removed and any blood that may remain on the point is carefully wiped away. If the pipette contains too much blood the necessary adjustment is made by gently tapping the point against the thumb nail when a very little blood will escape. If the skin and pipette are clean there is never any difficulty in obtaining the blood, and no particular speed is necessary. Indeed, if the first prick of the needle does not furnish enough blood, the rubber tube may be removed and again wound on and the operation repeated as often as three times or more without any fear of coagulation taking place. Sometimes it may be necessary to squeeze the finger, but since this does not appear to interfere with the result there is no objection to doing so. If the hand is cold it should be placed in hot water for a short time before taking the blood; also, the limb should be held in a hanging position for a few seconds before applying the rubber tube.

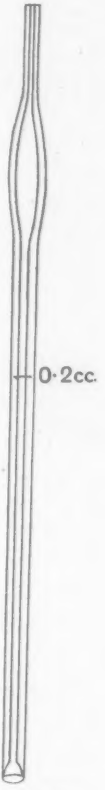

Fig. 2.

The one important point to be observed in using the method is that the *pipette should be kept scrupulously clean*; if any trace of greasy material is present on the inner surface the blood

[1] This pipette can be obtained from Hawksley & Sons, 357, Oxford Street, London.

will not flow. It is, therefore, most important that after each experiment the pipette should be washed out with a hot mixture of sulphuric acid and potassium bichromate. After thorough treatment with this mixture the pipette is washed out with distilled water followed by alcohol and ether and then dried. Personally, after each experiment I place the pipette in a test tube containing the bichromate mixture and let it stand there until it is again required when it is treated as described. When these precautions are taken there is never any difficulty in getting the blood.

Separation of protein from blood and estimation of sugar.

From a burette 23·8 cc. of acid sodium sulphate solution are introduced into a conical Erlenmeyer flask of heat-resisting glass. To this the 0·2 cc. blood is added and the pipette thoroughly washed out by alternately sucking in and blowing out the mixture. The blood is then heated and treated exactly as in the 1 cc. method, only that 1 cc. of dialysed iron is added instead of 3 cc. This gives a total volume of 25 cc. After cooling and filtering through a 9 cm. Whatman filter paper No. 1, 20 cc. of the filtrate are taken for the estimation of the sugar. In filtering, the process must be allowed to go on until all the material has passed through the filter since only about 21 to 21·5 cc. can be obtained altogether. In diabetic cases 10 cc. of the filtrate are taken and made up to 20 cc. with acid sodium sulphate solution.

To this filtrate are added 2 cc. (carefully measured) of the alkaline copper solution and boiling is carried out exactly as before for 6 minutes using a flame that brings the solution to brisk boiling in about 1 minute 40 seconds. It is then cooled thoroughly, 2 cc. of 75 % HCl added and the further treatment carried out exactly as described for the 1 cc. method except that $N/400$ thiosulphate is used for titration. The end point even with this dilute thiosulphate is perfectly distinct and gives no trouble whatever. The number of cc. of thiosulphate used is then subtracted from the number used by 2 cc. of copper solution alone, and the percentage of sugar in the blood is then calculated from Table II. Thus, if the reading obtained on titration was 8·98 cc. thiosulphate and if 2 cc. copper solution alone gives 11·05 cc., the difference (2·07 cc.) would represent the sugar present. From the table it is seen that 2·07 cc. thiosulphate = 0·18 mg. sugar; therefore $\frac{4}{5}$ (the aliquot part of filtra e taken) of 0·2 cc. blood contains 0·18 mg. glucose. 1 cc. of blood will therefore contain $\frac{1}{1} \times \frac{5}{4} \times \frac{5}{1} \times \frac{0{\cdot}18}{1}$ mg. glucose = 1·12 mg. = 0·112 %.

This "micro-method" gives excellent results with duplicates as the fol lowing numbers show. In blood from the finger the average sugar percentage appears to be somewhat higher than in blood taken from a vein. On an average 0·11 % may be taken to represent the normal. This is probably dependent on the fact that a good deal of the finger blood is capillary in origin and thus differs from the venous sample.

Table II.

(For estimation of sugar in 0·2 cc. blood.)

Showing equivalent of glucose to N/400 sodium thiosulphate solution.

Glucose	N/400 Thiosulphate cc.	Glucose	N/400 Thiosulphate cc.
0·03	0·12	0·22	2·61
0·04	0·25	0·23	2·74
0·05	0·38	0·24	2·86
0·06	0·50	0·25	2·99
0·07	0·62	0·26	3·11
0·08	0·73	0·27	3·24
0·09	0·86	0·28	3·36
0·10	0·99	0·29	3·49
0·11	1·13	0·30	3·61
0·12	1·26	0·31	3·74
0·13	1·39	0·32	3·87
0·14	1·53	0·33	3·99
0·15	1·67	0·34	4·12
0·16	1·80	0·35	4·24
0·17	1·94	0·36	4·37
0·18	2·07	0·37	4·49
0·19	2·22	0·38	4·62
0·20	2·35	0·39	4·74
0·21	2·49	0·40	4·87

Results of duplicate experiments on blood.

No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
0·112 %	0·127 %	0·114 %	0·079 %	0·066 %	0·041 %
0·116 %	0·126 %	0·119 %	0·084 %	0·069 %	0·038 %

The first three samples were obtained from the finger, one sample being taken immediately after the other. The very close results obtained indicate the accuracy of the method. Experiments 4, 5 and 6 were done on three different bloods obtained from a vein; they had stood in the laboratory for varying periods, hence the low results.

The question of the apparent difference in sugar content between capillary and venous blood is at present being investigated; also the effect of different food stuffs and starvation on the blood sugar.

Summary.

A simple method is described by which an accurate estimation of the sugar present in 1 cc. and in 0·2 cc. of blood may be estimated. The method requires careful standardisation of the heating, but otherwise no special care is necessary. With pure glucose solutions theoretical results are obtained and in blood the duplicates always agree within very narrow limits.

REFERENCES.

Bang (1918). *Biochem. Zeitsch.*, **87**, 248.
Cole (1914). *Biochem. J.*, **8**, 134.
De Wesselow (1919). *Biochem. J.*, **13**, 148.
MacLean (1916). *J. Physiol.*, **50**, 168.
Scales (1915). *J. Biol. Chem.*, **23**, 81.

XVII. THE PICRIC ACID METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD AND A COMPARISON OF THIS METHOD WITH THAT OF MACLEAN.

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(*Received April 16th, 1919.*)

The colorimetric method for the estimation of sugar in the blood as originally introduced by Lewis and Benedict [1915] has recently been modified by Benedict [1918], and considerably simplified. Addis and Shevky [1918], writing previous to the publication of Benedict's paper, have pointed out that the colour produced by the picric-sugar reaction is not strictly proportional in intensity to the amount of sugar present. Lewis and Benedict in their original paper state that in estimating pure sugar solutions absolutely exact results were obtained, using different amounts of sugar against the same standard, but the work of Addis and Shevky would appear to negative this conclusion. That the latter observers are correct in their statement admits of no doubt and this difference was observed by me before I knew of their paper. Addis and Shevky ascertained that the nearest approach to an exact proportion between intensity of colour produced and amount of sugar present was obtained when an amount of sodium carbonate sufficient to give a concentration of 10 % was present, and the heating was continued for 45 minutes at 100°. Such conditions are not realised in Benedict's modification and in estimations of pure sugar solutions by this method a considerable error is in fact found. In the estimation of sugar in normal bloods, however, the direct error would be of no great magnitude since the sugar content of such bloods approaches closely to that of the standard.

The second possible source of error, the presence of interfering substances in the blood, was also dealt with by Addis and Shevky. They prepared large amounts of filtrates from hyperglycaemic bloods, and, comparing the curves obtained from these under different conditions of alkali concentration and temperature with those obtained from dextrose solutions, came to the conclusion that no interfering substances were present. In these experiments, however, the heating appears to have been prolonged for 45 minutes, no readings having been carried out during the very early stages of the heating.

Benedict replying to the verbal criticism that the picric acid method yields too high results for blood owing to colour production by non-carbohydrate substances gives figures for five samples of dog's blood, tested by the picric acid method before and after treatment of the blood with mercuric nitrate. In two of these samples the apparent sugar content fell after treatment with mercuric nitrate by 19 % and 21 % respectively.

In view of the above possibilities of error in the Benedict method a direct comparison with another method of sugar estimation—that of MacLean [1916, 1919]—appeared advisable. The directions of Benedict were exactly followed, a sugar standard freshly prepared from Kahlbaum's pure glucose being used. On comparing the results obtained in human blood it was found that the Benedict method invariably gave a considerably higher percentage of sugar than that of MacLean. Since Lewis and Benedict [1915] in their original paper noted that haemolysis previous to the addition of the picric solution raised considerably the estimated percentage of sugar, the sugar of the whole blood and of the plasma was estimated in a series of human bloods by both methods with the following results.

	Whole blood. Sugar %		Plasma. Sugar %	
No.	Benedict	MacLean	Benedict	MacLean
(1)	0·114	0·089	0·103	0·099
(2)	0·137	0·104	0·118	0·113
(3)	0·130	0·081	0·112	0·090
(4)	0·176	0·139	0·167	0·147
(5)	0·155	0·092	0·113	0·102
(6)	0·132	0·082	0·114	0·090
(7)	0·157	0·099	0·135	0·110
(8)	0·100	0·067	0·069	0·070

It appears that the sugar content of the whole blood as estimated by the Benedict method is always considerably higher than the result given by the method of MacLean—the excess averaging about 45 % of the sugar present. On the other hand the Benedict results for plasma approximate closely to those obtained by the MacLean method, the excess amounting on an average to about 12 %. While by the MacLean method, the whole blood shows a slightly lower sugar content than the plasma, the Benedict estimation shows a very considerable excess of sugar in the whole blood as compared with the plasma. The difference between the results of the two methods is brought out more strongly if the blood is centrifuged or allowed to sediment in the ice chest and the plasma and corpuscular layers investigated separately.

	Plasma. Sugar %		Corpuscles. Sugar %	
No.	Benedict	MacLean	Benedict	MacLean
(1)	0·108	0·082	0·143	0·064
(2)	0·092	0·079	0·129	0·057
(3)	0·080	0·082	0·113	0·062

Under these conditions while the plasma gives an excess of sugar of 12 % by the Benedict method, the sediment richly laden with corpuscles shows an

average excess of no less than 66 % over the MacLean method. Again, the portion of the blood containing the corpuscles gives a considerably higher figure for sugar than the plasma, while with the method of MacLean, as might be expected from the greater solid content of the corpuscles, less sugar is found in the corpuscular sediment than in the plasma. It should be noted that the figures obtained above do not necessarily represent the original con tent of sugar in the blood, for several of the specimens had stood in the labora tory for some time before the estimation was carried out.

These results appeared to suggest the presence of an interfering substance in the corpuscles giving a colour reaction with picric acid and raising the supposed sugar content of the whole blood. To some extent they paralle the results obtained by Hunter and Campbell [1917] in creatinine estimations by the Folin method.

A consideration of the very different results obtained by the two methods in whole blood or in blood corpuscles indicates that one of the methods must give entirely fallacious results. If, for purposes of argument, we assume tha the error lies in MacLean's method we are forced to accept the extraordinary view that blood corpuscles contain some substance which prevents the sugar present from reducing its equivalent amount of copper when heated in alkaline copper solution. That such a view is untenable is proved by the fact that in the case of blood which has been incubated for a period sufficiently long to allow glycolysis to destroy all the sugar present, any sugar which is added can be recovered quantitatively. If the blood corpuscles contained a substance which interfered with this method, it is difficult to understand why they should not prevent the added sugar from reacting in the normal way. On the other hand a blood so treated gives by Benedict's method a greater percentage of sugar than has been added. Instead of giving values of too low an order, it is highly probable that MacLean's method tends if anything to yield results slightly on the high side, and the different values obtained in the two methods cannot be explained on the supposition that MacLean's method really gives low results.

To prove however that Benedict's results are too high, experiments were carried out to determine whether any direct evidence of the presence of inter-fering substances could be found in the blood.

If the whole colour reaction was due to sugar, identical readings against a standard sugar solution should be obtained at any stage of the reaction. To test this point samples of the same blood filtrate were heated with a sugar standard for short periods of time, cooled, and rapidly read on the colorimeter. The standards contained as nearly as possible the same amount of blood sugar as the filtrates. Samples of plasma and sedimentated corpuscles were also examined. The concentrations of picric acid and alkali were the same in the sugar standard as in the filtrates to be tested. The results obtained were plotted as curves (Fig. 1). It is obvious from these curves that, while the plasma gives only a slight though distinct variation, the whole blood gives *high initial*

readings falling sharply with prolongation of the heating, and the sedimented corpuscles give even higher readings at the outset with a similar rapid fall. The only possible explanation of this result is the presence of a substance differing from glucose and mainly concentrated in the corpuscles which reacts with picric acid solution more rapidly than sugar, and by its additive effect leads to the ultimate high readings obtained by the Benedict method. This substance is present to a small extent only in the plasma, and consequently estimations carried out on plasma by the Benedict method in normal bloods show only slight excess over those obtained by the method of MacLean. In hyperglycaemic bloods even the results obtained from plasma are likely to be unsatisfactory, owing to the lack of direct proportion between the intensity of colour produced and sugar present under the conditions of the test.

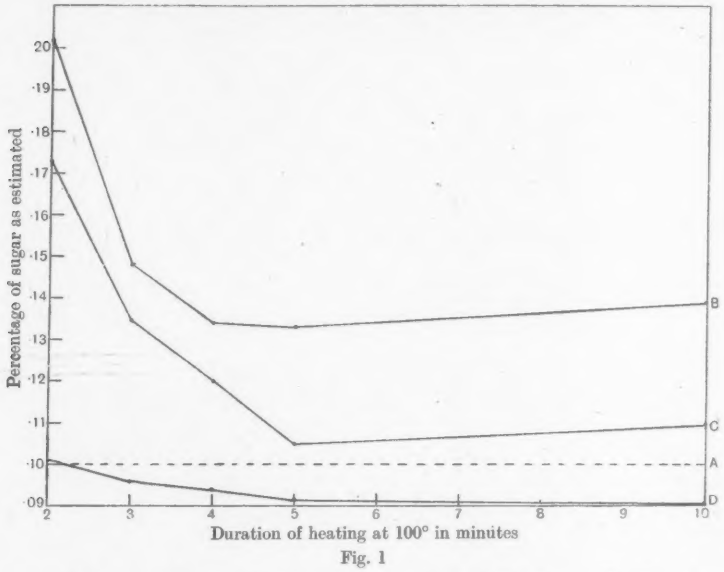

Fig. 1

A glucose standard. B corpuscles C whole blood. D plasma.

Conclusions.

1. The Benedict method for the estimation of sugar in blood gives results which are too high and shows an average figure about 30 to 50 % in excess of that found by MacLean's method.

2. The high results appear to be chiefly due to the presence of an interfering substance or substances mainly concentrated in the corpuscles but present to some extent in the plasma also; this substance reacts with the picric solution at an early stage of the heating. Creatinine probably plays a large part in this reaction.

3. On account of the influence of this interfering factor the accurate estimation of sugar in whole blood by the picric acid method as described by Benedict is impossible.

REFERENCES.

Addis and Shevky (1918). *J. Biol. Chem.*, **35**, 43.
Benedict (1918). *J. Biol. Chem.*, **34**, 203.
Hunter and Campbell (1917). *J. Biol. Chem.*, **32**, 195.
Lewis and Benedict (1915). *J. Biol. Chem.*, **20**, 61.
MacLean (1916). *J. Physiol.*, **50**, 168.
—— (1919). *J. Biol. Chem.*, **13**, 135.

XVIII. ENTEROINTOXICATION—ITS CAUSES AND TREATMENT.

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(*Received April 28th, 1919.*)

The function of the intestinal flora and the relationship of the flora to such of its products as are absorbed by the organism have been matters of some controversy.

Two conflicting views have been held, viz.

(1) That the intestinal microbes are useful to the organism.

(2) That the intestinal microbes are harmful to the organism.

The first hypothesis is nothing more than a teleological conception without supporting evidence; the second is based on observations, especially as to the relationship between the intestinal flora and the absence of urinary indican in the breast-fed child.

The contention has hitherto been left undecided owing to the lack of crucial experiments which alone are satisfactory for the elucidation of biological problems.

The present contribution deals shortly with experiments performed with a view to demonstrating that indican and other products in urine are due to the activity of intestinal microbes.

To put this conclusion on an unshakable basis it is necessary to demonstrate that *in vivo* an intestinal flora containing predominantly indole-forming micro-organisms gives rise to indican in the urine, whereas by transforming at will such a flora into one containing in predominance non-indole forming micro-organisms, the indican and other products disappear from the urine.

The first stage in the investigation of the relationship between intestinal flora and urinary poisons is to find a method of transforming the flora. This was done by feeding white rats upon a food diet which included lactose [Distaso and Schiller, 1914]. After feeding for two or three days with this diet the faeces acquire an acid reaction and a yellow colour. The predominant microbe is just the same as in breast-fed infants, viz. the *B. bifidus communis* which never produces indole. We were led to adopt this method starting from the hypothesis that certain enzymes may be absent in adult rats which would result in a sugar passing untouched into the caecum, and so into the large intestine, whereby microbes of what one of us has called *the acetogenous group* (*B. bifidus, acidophilus*, etc.) would be able to grow [Distaso, 1911].

Under these conditions just as is the case in experiments *in vitro* the higher the percentage of sugar available for the intestinal microbes the more quickly the acetogenous flora gain the ascendency, and afterwards they are the only species which survive. We dismissed, therefore, the idea of introducing new microbes into the intestine because in each intestinal flora there are plenty of acid producers and non-indole forming organisms which are always ready to seize the first opportunity for displaying activity. Moreover we found to our astonishment that we were unable to acclimatise strange microbes to the environment of the intestinal flora even though the most favourable conditions were provided [Distaso and Schiller, 1914].

The discovery of this method opens up a wide field from the physiological and pathological standpoint. The rapid sensibility of the intestinal flora to such influence should certainly permit us to solve the question of the relationship between intestinal flora and the urinary poisons, and to acquire a more exact knowledge as regards the activity of the enzymes of the digestive canal in each animal species under normal and pathological conditions and assuredly supplies a more direct and trustworthy method of investigation than any optical method can.

At any rate one problem in connection with the nutrition of the breast-fed child seems to be explained in connection with this discovery. Certainly in this case a sugar is prepared in the breast of the mother, and this is not absorbed by the young organism, but passes untouched into the large intestine. Why the mother prepares in her breast something which is of no nutritive value to the suckling is one of the riddles which it is hard to understand if one carefully avoids teleological explanations.

Relationship between Indole and Microbes.

In this connection, the first point which can be demonstrated *in vitro* is that the indole, of which the indican is the oxidation product, is formed by the activity of the microbe alone. In fact if proteins are really split by sterile methods in the presence of intestinal juice, it will be observed that the products of the cleavage reach the tryptophan stage. If the same process is carried out in the presence of microbes, indole will be at once detected. The indole is, therefore, a product of the activity of the microbes, and is not formed by the enzymes of the digestion. This conclusion is supported by Nencki's classical experiments *in vivo*. He found that the products of digestion drawn off through an iliac fistula never contain a trace of indole, whereas it is easy to detect indole in the faeces where we know the microbes display all their activity. Thus *in vitro* as well as *in vivo* it has been demonstrated that indole is formed by the activity of the microbe.

We now proceed to give below some of our experiments which demonstrate that indican disappears from urine if we cause the indole forming flora to disappear, whereas it reappears as soon as the indole forming flora reappears.

We have, moreover, dealt with the question of the production of ethereal sulphates and skatoxyl and we believe there is evidence enough of the mechanism and source of their production.

The feeding experiments were made at intervals over a considerable period, under varying conditions with regard to diet and amount of lactose used. In all cases control feeding experiments were made at the same time as the lactose feeding tests, using groups of from one to three animals as available. The amount of food given (other than lactose) was not weighed, but the animals were of approximately equal size, and were supplied with the food in approximately equal amounts.

Putrefaction of the urine was inhibited by admixture with small amounts of suitable antiseptic.

It was necessary at times to continue the collection for a period of 48 hours or more, although, had the conditions permitted, it would have been more satisfactory to have been able to make analyses upon the freshly collected samples.

Analytical Results.

Indoxyl. The figures given under this term may be regarded as approximating to the indoxyl present, since the colouring matter was formed in the presence of an oxidising agent, and therefore indigo blue should be the chief product and its amount be approximately in proportion.

10 cc. of urine previously treated with basic lead acetate and filtered, were mixed with an equal volume of pure strong HCl and extracted with 2 cc. of chloroform, adding 1–2 drops of 1 in 10 H_2O_2.

A second extraction was made where any considerable development of indigo resulted. The intensity of colour was then matched against a standard tint on an arbitrary scale. No great degree of accuracy can be attained by the method and the figures are merely relative, but they serve for purposes of comparison.

Amyl Alcohol Extract.

The residual acid liquid was extracted once or twice with small amounts of amyl alcohol, and as with the chloroform extract the coloration obtained was recorded on an arbitrary scale.

To what extent these figures give a measure of the skatoxyl pigment is a matter of controversy; also the tints were not clearly defined and colorimetric readings were more indefinite than with the blue chloroform extracts.

Ethereal Sulphates.

These determinations as well as those of the mineral sulphate were made by the benzidine method as described by Rosenheim and Drummond. The figures are given in terms of milligrams SO_3 per animal per diem. They will

include not only the indoxyl sulphates but also any skatoxyl sulphates, phenol sulphates, etc. It will be found that the few determinations which could be made show some relationship (as is to be anticipated) to the figures for indoxyl.

We give below a few typical experiments for the sake of brevity.

Experiment 1.

Control—Potato
Lactose—Potato + 2·5 g. lactose per animal.
From October 30 to November 16, 1916.

Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
1916					
Nov. 3	Control ...	10·5	0·6	6·9	0·6
Nov. 6	,, ...	3·5	0·8	7·3	0·3
Nov. 10	,, ...	Nil	—	5·0	Nil
Nov. 12	,, ...	1·0	—	6·1	Nil
Nov. 16	,, ...	Nil	—	2·3	Nil
Nov. 5	Lactose 2·5 g.	Nil	0·1	—	—
Nov. 11	,, ...	Nil	—	2·7	Nil
Nov. 16	,, ...	Nil	—	1·5	Nil

Remarks. The continued feeding of the control animals with potato only shows a gradual reduction in the figures for indoxyl and also for ethereal sulphates which after two weeks were practically nil.

Although the lactose feeding experiment shows the rapid disappearance of indoxyl and other products within a few days, the experiment is in our opinion inconclusive.

We have been obliged to select another diet which would give a greater and a persistent amount of indican, skatoxyl, ethereal sulphates, etc., in the control, a condition under which alone a crucial experiment would assume its full value.

Experiment 2.

Control—Banana.
Lactose—Banana + 2·5 g. lactose per animal.
From March 14 to March 30, 1917.

Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
March 19	Control ...	Traces	2·0	—	—
March 22	,, ...	1·5	2·2	0·25	0·1
March 27	,, ...	2·4	0·4	0·3	0·1
March 30	,, ...	1·9	1·2	0·15	0·05
March 20	Lactose 2·5 g.	0·4	0·2	—	—
March 23	,, ...	Nil	0·2	0·15	Nil
March 27	,, ...	Nil	1·0	0·1	Nil
March 30	,, ...	Nil	0·8	—	—

Remarks. In the control feeding the figures for ethereal sulphates (also mineral sulphates) were exceptionally low throughout, but they are capable of comparison.

Lactose feeding gave negative results within a few days for both indoxyl and ethereal sulphates, but the amyl alcohol extract continues to be appreciable in the lactose-fed animals.

Experiment 3.

Control—Expt. 2 Lactose animals now fed with banana.
Lactose—Expt. 2 Control animals now fed with banana + 2·5 g. lactose per animal.
From March 30 to April 23, 1917.

Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
April 2	Control ...	Nil	1·6	0·12	Nil
April 4	„ ...	1·1	0·5	0·14	Nil
April 6	„ ...	7·0	0·7	0·09	0·03
April 7	„ ...	2·0	0·5	—	—
April 10	„ ...	1·0	1·5	—	—
April 17	„ ...	1·2	0·6	—	—
April 19	„ ...	3·0	1·0	—	—
April 23	„ ...	2·0	1·0	—	—
April 3	Lactose 2·5 g.	Nil	0·3	—	—
April 7	„	Nil	0·3	—	—
April 10	„	Nil	Prac. Nil	—	—
April 13	„	Nil	„	—	—
April 17	Banana only	Nil	„	—	—
April 20	„	0·5	0·5	—	—
April 23	„	0·5	0·5	—	—

Remarks. Feeding reversed. On feeding the original control animals with lactose, negative results for indoxyl were obtained within a few days. The original lactose-fed animals (now banana only) showed a gradual re-appearance of indoxyl.

The same phenomena were to be observed in this experiment as in the previous one; with the appearance of indican, the flora showed chiefly coliform and allied micro-organisms, whereas with its disappearance the acetogenous group partially re-appeared.

We attribute the persistence of the figures for amyl alcohol extract to the fact that it is almost impossible to mix thoroughly the lactose with banana. Further, the transformation of the intestinal flora thereafter was not complete, smears made from films actually showing an appreciable proportion of *B. coliformis.*

Experiment 4.

May 24 to June 8.
Control—Bread for two days previously, then bread and egg.
Lactose—Bread for two days previously, then bread and egg* + lactose: 4 g. to June 2; 5 g. to June 6; 8 g. to June 8 per animal.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
May 24	Control ...	14·0	Small amount	—	—
May 27	,, ...	22·0	Fair amount	—	—
May 29	,, ...	32·0	4·0	—	—
May 31	,, ...	16·0	1·6	12·5	1·2
June 2	,, ...	24·0	1·5	—	—
June 4	,, ...	28·0	2·0	—	—
June 6	,, ...	32·0	2·0	—	—
June 8	,, ...	22·0	2·2	24·1	2·8
May 24	Lactose 4·0 g.	12·0	Small amount	—	—
May 27	,,	10·0	Fair amount	—	—
May 29	,,	6·0	0·8	—	—
May 31	,,	3·0	0·4	4·6	0·25
June 2	,,	2·4	< 1·0	—	—
June 4	,, 5·0 g.	< 0·05	0·5	3·4	0·5
June 6	,,	Prac. nil	1·0	4·9	0·5
June 8	,,	0·8	0·4	4·3	0·3

* This was prepared as follows: an egg was beaten and diluted with 4 times its volume of water. The bread was soaked in this emulsion.

Remarks. The control feeding gave very high figures for indoxyl. The lactose feeding experiment shows a gradual reduction in indoxyl, ultimately to nil, with a large reduction in ethereal sulphates.

The experiment was stopped at this stage, because the rats began to refuse their food and, as will be seen later, this antipathy towards lactose has been an adverse factor to cope with, which has required special conditions of feeding.

Experiment 5.

June 14 to July 16.
Control—Bread and egg; fed on bread for six days previously.
Lactose—Bread and egg + 8 g. lactose per animal up to June 22.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
June 14	Control ...	3·0	—	—	—
June 16	,, ...	13·0	Fair amount	—	—
June 18	,, ...	30·0	,,	—	—
June 20	,, ...	17·5	—	11·3	0·7
June 22	,, ...	12·0	1·8	—	—
June 29	Bread only	18·0	Small amount	—	—
June 30	,, ...	13·0	Fair amount	—	—
July 11	,, ...	17·5	,,	—	—
July 16	,, ...	16·0	,,	—	—
June 14	,, ...	7·0	—	—	—
June 16	Lactose 8 g.	1·5	Small amount	—	—
June 18	,, ...	0·2	,,	—	—
June 20	,, ...	0·3	—	0·2	Practically nil
June 22	,, ...	0·4	Nil	—	—
June 27	Bread only	1·6	Traces	—	—
June 30	,, ...	1·6	< 0·8	—	—
July 11	,, ...	4·5	Small amount	—	—
July 16	,, ...	10·0	Apprec. amount	—	—

Remarks. The control feeding showed high figures for indoxyl and these were still maintained on a reversed diet of bread only. In the lactose feeding a quick reduction was obtained in both ethereal sulphates and indoxyl to practically nil. The indoxyl gradually re-appeared after stopping feeding with lactose, reaching the normal amounts in about three weeks.

Experiments 4 *and* 5.

Although in these experiments we have been unable to reduce the amounts of indican, amyl alcohol extract and ethereal sulphate to nil, this partial failure is, in our opinion, due to certain adverse circumstances, *viz.*

(1) The diet was really very severe.

(2) We have been obliged to use old animals which owing to long experience had become unsuitable for use.

(3) The rats have a distaste for lactose.

In order to avoid a condition of starvation in this and in other experiments they were discontinued whenever these adverse factors were becoming acute.

Experiment 6.

July 23 to August 18.

Control—Fed on bread three days previously, then bread and egg.

Lactose—Fed on bread three days previously, then bread and egg to July 26, then 8 g. lactose, then 5 g., then 10 g. lactose per animal.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
July 23	Control ...	28·0	Apprec. amount	—	—
July 25	„ ...	20·0	1·5	13·4	1·0
July 28	„ ...	19·0	1·7	—	—
July 31	„ ...	33·0	3·2	—	—
Aug. 3	„ ...	16·0	1·1	14·3	0·7
Aug. 6	„ ...	27·0	1·5	—	—
Aug. 10	„ ...	Large amount	1·1	—	—
Aug. 13	„ ...	21·0	1·8	16·4	1·1
Aug. 15	„ ...	10·0	Small amount	—	—
Aug. 18	„ ...	9·0	Fair amount	—	—
July 23	Bread ...	32·0	Fair amount	—	—
July 25	Bread and egg	19·0	1·9	13·2	1·0
July 28	Bread and egg + Lactose 8 g.	< 1·0	0·3	—	—
Aug. 1	„	0·4	0·2	—	—
Aug. 3	„ 5 g.	0·6	0·5	6·8	Nil
Aug. 6	„	0·4	Practically nil	—	—
Aug. 10	„	Trace only < 0·05	0·2	—	—
Aug. 13	„ 10 g	0·3	Practically nil	0·2	Nil
Aug. 15	„	2·0	Traces	—	—
Aug. 18	„	4·2	Apprec. amount < control	—	—

Remarks. The control feeding showed high figures for indoxyl which were reduced towards the end of the period. With lactose feeding the amounts of both indoxyl and ethereal sulphates were quickly reduced to almost nil and finally also the mineral sulphates, but afterwards the indoxyl began to reappear.

This is an illustrative experiment in which the results obtained may appear to indicate a failure on our part to produce systematically and always the disappearance of urinary poisons. But the flora in this case suggests the explanation. Here it is to be noted that there is an enormous fall in urinary poisons after three days of lactose feeding, but at the same time the flora was far from showing the homogeneity seen in other cases. This condition had continued for 13 days, when we decided to increase the amount of lactose. Now the rats took little food, it being almost entirely rejected—a condition of starvation in which all the classical symptoms ensued and which led to a definite increase in the urinary poisons. The flora on the other hand again returned to the condition of the control flora, viz. predominance of *B. coliformis*.

Experiment 7.

September 17 to October 7, 1917.
Control—Bread and egg.
Lactose—Bread and egg + 8 g. lactose per animal.

	Test	Indoxyl	Amyl Alcohol Extract
Sept. 17	Control Bread ...	6·0	1·2
Sept. 19	,, Bread + egg	6·5	1·9
Sept. 21	,, ,,	8·0	2·6
Sept. 24	,, ,,	12·0	2·3
Sept. 26	,, ,,	10·0	1·8
Sept. 29	,, ,,	7·2	1·3
Oct. 1	,, ,,	14·5	1·2
Oct. 4	,, ,,	13·0	1·3
Oct. 7	,, ,,	13·0	0·5
Sept. 17	...	5·5	1·4
Sept. 19	Lactose 8 g. ...	2·0	0·5
Sept. 21	,, ...	1·0	0·3
Sept. 24	,, ...	2·2	0·4
Sept. 26	,, ...	1·6	0·4
Sept. 29	,, ...	1·2	0·6
Oct. 1	,, ...	1·1	0·7
Oct. 4	,, ...	1·5	1·0
Oct. 7	,, ...	0·5	0·5

Remarks. Old rats used many times. The control feeding gave indoxyl in fair amount, but less than in earlier experiments. With the lactose feeding a slow gradual reduction in amount of indoxyl is to be noted. A certain amount of indican, although small, persisted to the end of the experiment, and an observation of interest is that the flora always contained a certain number of Gram-negative bacilli (*B. coliformis*).

As previously stated, the rats were very fastidious in choosing their food, selecting the crumbs and rejecting lactose so far as possible, and of course without lactose introduced into the intestine the experiment will fail.

It is very difficult to secure a thorough admixture of lactose with bread and therefore for later experiments we chose cooked potatoes to which an emulsion of egg was added.

Experiment 8.

Control—Potato and egg.
Lactose—Potato and egg + 8 g. lactose per animal.
Nov. 14 to Nov. 27, 1917.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
Nov. 14	Control	8·5	—	—	—
Nov. 20	,, ...	8·5	Trace only	—	—
Nov. 24	,, ...	5·0	Practically nil	—	—
Nov. 27	,, ...	8·0	,,	10·4	0·6
Nov. 14	Before Lactose ...	8·4	—	—	—
Nov. 20	+ Lactose 8 g. ...	0·2	—	—	—
Nov. 24	,, ...	Nil	0·3	—	—
Nov. 27	,, ...	Nil	Practically nil	7·3	Nil

Remarks. The control feeding showed indoxyl and ethereal sulphates in fair amount, less than in the earlier feeding with bread and egg, but the amounts were persistent throughout the experiment. With the lactose feeding a rapid elimination of indoxyl occurred with the practical elimination of ethereal sulphates within ten days.

Experiment 9.

Control—Potato and egg.
Lactose—Potato and egg + 8 g. lactose per animal.
December 18, 1917, to January 7, 1918.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
Dec. 20	Control	8·5	Practically nil	—	—
Dec. 22	,, ...	5·0	0·6	—	—
Dec. 24	,, ...	4·5	0·7	8·4	0·9
Dec. 25	,, ...	Fair amount	Small amount	—	—
Dec. 31	,, ...	4·5	,,	—	—
Jan. 2	,, ...	7·4	,,	—	—
Jan. 4	,, ...	7·8	1·3	18·0	0·8
Jan. 7	,, ...	8·0	Nil	14·5	1·9
Dec. 20	Lactose 8 g. ...	0·2	Practically nil	—	—
Dec. 22	,, ...	Nil	Nil	—	—
Dec. 24	,, ...	Nil	Nil	—	—
Dec. 28	,, ...	Nil	Nil	—	—
Dec. 31	,, ...	Nil	Nil	—	—
Jan. 2	,, ...	Nil	Nil	—	—
Jan. 4	,, ...	Nil	Nil	8·3	Nil
Jan. 7	,, ...	Nil	Nil	12·3	Nil

Remarks. The control feeding results were similar to those in Experiment 8, whilst the lactose experiment also gave results practically identical with the previous Experiment 8. With this diet we have, therefore, reached the ideal experimental conditions for proving our assertion and in this and the following experiment it will be seen that with these conditions realised the experiments will give consistently the same results.

Experiment 10.

Control—Potato and egg.
Lactose—Potato and egg + 8 g. lactose* per animal.
March 12 to 26, 1918.

	Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_4
March 18	Control	3·4	1·2	—	—
March 19	„	8·4	2·0	24·0	2·2
*March 20	„	5·4	Small amount	—	—
*March 22	„	9·6	„	15·9	2·45
*March 23	„	12·8	„	—	—
March 25	„	19·0	„	16·3	2·0
March 26	„	17·0	Yellowish	—	—
March 18	Lactose 8 g. ...	Nil	Nil	—	—
March 19	„ ...	Nil	Nil	—	—
March 20	„ ...	Nil	Nil	—	—
March 22	„ ...	Nil	Nil	—	—
March 23	„ ...	Faint trace	—	—	—
		< 0·1	Practically nil		
March 25	„ ...	Nil	Nil	7·7	Nil
March 26	„ ...	Nil	Nil	13·2	Nil

* Since under the conditions of the experiments some direct admixture of lactose with the urine was unavoidable, an amount of lactose approximately equal to that given in the food was in these control tests added to the containing vessels, giving a direct admixture of the urine with lactose with no marked fluctuations in the results obtained.

Remarks. In the control feeding the amounts of indoxyl and ethereal sulphates reached a higher figure than with previous experiments. The lactose experiment showed a quick elimination of indoxyl and also of ethereal sulphates.

Conclusions.

We have been able to show that the presence of indoxyl, skatoxyl and ethereal sulphates in the urine, corresponds with a flora composed of microbes which *in vitro* give indole and skatole.

On the other hand if such a flora be transformed into one which does not produce either indole or skatole, then indoxyl, skatoxyl and ethereal sulphates are absent from the urine.

The group of microbes which produces these poisons is that of *B. coliformis* which play a great rôle in many intestinal disorders and states of ill-health, and to produce disintoxication the intestinal flora must be transformed so as to consist of non-indole producers.

It is interesting to note that throughout our experiments the total mineral sulphates are lower in the non-intoxicated animals, and this observation should open up an experimental field of considerable importance in relation to the metabolism of the mineral constituents of the animal body.

We admit that the amount of mineral matter in the food used will influence the excretion of the mineral sulphates, but nevertheless our experiments show throughout a marked decrease of the total mineral sulphates with lactose feeding.

This effect we attribute to the fact that in intestinal stasis and in cases where intestinal fermentation is abnormal, the food is far more broken down by the action of the microbes and therefore more absorption of the mineral products takes place, with greater excretion in the urine.

REFERENCES.

Distaso (1911). *Centralb. Bakt. Par.* I Orig., **59**, 48.
Distaso and Schiller (1914). *Compt. Rend. Soc. Biol.*, **1**, 179, 243.

XIX. THE ACTION OF ULTRA-VIOLET RAYS ON THE ACCESSORY FOOD FACTORS.

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(*Received April 28th, 1919.*)

In spite of the various attempts to isolate and identify the accessory food factors their chemical identity still remains unsolved. As far as the antineuritic and the antiscorbutic factors are concerned it is possible now by means of chemical fractionation to obtain residues which are very active. These preparations contain comparatively little impurity judging from the small quantities required to produce a therapeutic effect. Although the chemical aim has not been achieved the possibility of employing such active fractions has been found useful in furthering research on the accessory factors in the domain of nutrition.

As the chemical identification of these principles has not so far been successful by means of fractionating methods it becomes advisable to study their properties, because it contributes to the knowledge of the nature of these unknown substances. During the various investigations carried out on the accessory factors many of their properties have already been revealed. So far, however, the action of light on them has not been studied. This investigation is devoted to the study of ultra-violet rays on the accessory factors. These rays are well known to produce pronounced chemical changes in many compounds and also to influence the physiological activity of various biological principles, and it was therefore of interest to find out the effect on the accessory factors after an exposure to ultra-violet rays. The action of the light on the antiscorbutic, antineuritic and the fat-soluble *A* factor was investigated. As these factors cannot be obtained in pure form they could only be exposed to the ultra-violet rays as present in active substances. The antiscorbutic was used in the form of lemon juice from which the citric and other acids have been removed as described by Harden and Zilva [1918]. Autolysed yeast was used for the antineuritic and butter was employed as the source of the fat-soluble *A*.

Experimental.

The Antiscorbutic Factor.

In using the treated lemon juice for the antiscorbutic factor it was brought to a definite H-ion concentration in order to equalise the conditions. This was done colorimetrically by the addition of *N*/10 HCl to the juice and matched against a standard solution of glycine and *N*/10 HCl estimated by the gas

chain method to be P_H 2·34. The H-ion concentration was adjusted daily before the liquid was exposed to the ultra-violet light. The solution was introduced in a quartz tube of 1 inch diameter which was revolved by means of a water motor. As a source of ultra-violet rays the quartz mercury vapour lamp was used and placed about 6 inches from the revolving tube. The solution was exposed to the light for 8 hours. Three sets of animals were employed in this experiment. One set received the treated lemon juice without having its reaction adjusted, the other set received the treated lemon juice with its reaction adjusted as described above and the third set received the treated lemon juice brought to P_H 2·34 and exposed for 6 hours to the ultra-violet rays. Each set had four animals which received 0·5 cc., 1·5 cc., 2·5 cc. and 5 cc. of the respective solutions. Fig. 1 represents the weight curves of these animals.

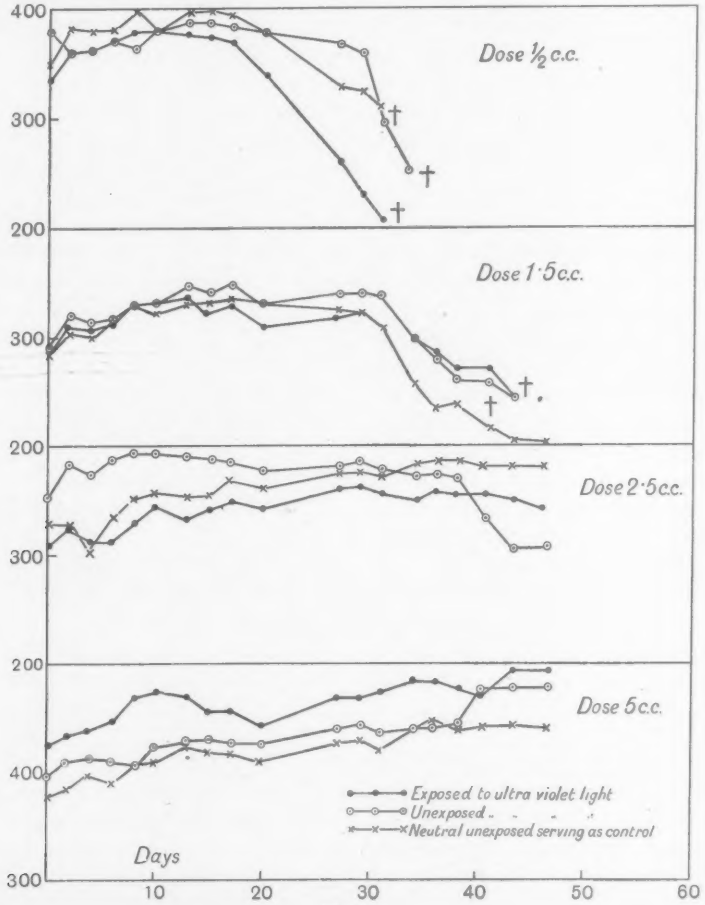

Fig. 1

The animals in all the sets which received 0·5 cc. of the solution succumbed to scurvy within about 30–35 days. Of the animals receiving 1·5 cc. of the various solutions two died of scurvy within 42 days, and the third one receiving the treated lemon juice as a control was decreasing rapidly in weight when it was chloroformed on the 46th day. The post mortem revealed very marked scurvy. The guinea pigs which received 2·5 cc. of the solution all survived the duration of the experiment, namely 46 days. These animals were chloroformed and at the post mortem examination no signs of scurvy could be detected. The dose of 5 cc. also warded off scurvy entirely in all cases. These animals were growing when they were chloroformed after they had been under experiment for 46 days. They were found in good condition and no traces of scurvy could be detected.

One is bound to conclude from the above experiment that the exposure to the ultra-violet rays did not destroy or even impair the antiscorbutic activity of the treated lemon juice at the H-ion concentration of P_H 2·34.

An attempt was then made to ascertain whether the action of ultra-violet rays has any effect on the treated lemon juice in neutral condition. Lemon juice treated by the Harden and Zilva method is neutral or slightly acid to litmus; this reaction depends on how thoroughly the organic acids have been removed from the lemon juice. By taking the necessary precautions it is possible to prepare it always so that it reacts neutral to litmus. Such neutral preparations of lemon juice were used in this experiment. One group of guinea pigs received the exposed treated lemon juice; to the other group the unexposed treated lemon juice of the same batch was administered. As in the previous experiment doses of 0·5 cc., 1·5 cc., 2·5 cc. and 5 cc. were given. In this case too both animals receiving 0·5 cc. declined and died of scurvy within about a month, thus showing 0·5 cc. of the treated lemon juice to be a dose insufficient to delay or prevent the onset of scurvy in guinea-pigs. The dose of 1·5 cc. in both cases delayed but did not prevent the onset of scurvy. The animal receiving the solution exposed to the ultra-violet rays was chloroformed and found to be suffering from acute scurvy. The guinea pig which received the control solution died of scurvy after 33 days. 2·5 cc. and 5 cc. of both solutions was sufficient to prevent scurvy. The weight curves of the above animals (Fig. 2) show that no great differentiation can be established in the growth of the animals receiving similar doses of the respective solutions. It is therefore evident that the action of the ultra-violet rays produced no marked deterioration of the antiscorbutic potency of the neutral treated lemon juice.

The Antineuritic Factor.

In studying the influence of ultra-violet rays on the antineuritic accessory factor pigeons and rats were employed. A pigeon was kept on a diet of polished rice until it developed polyneuritis; a dose of the exposed autolysed yeast was then administered in order to see whether it had a curative effect. The

autolysed yeast was exposed to ultra-violet light in a similar manner to the lemon juice. The following protocol describes the experiment in detail.

Pigeon *H* showed decided symptoms of polyneuritis. Its head was bent well forward. The animal was unable to walk and could not stand firmly on its legs. 3 cc. of the exposed autolysed juice was administered per

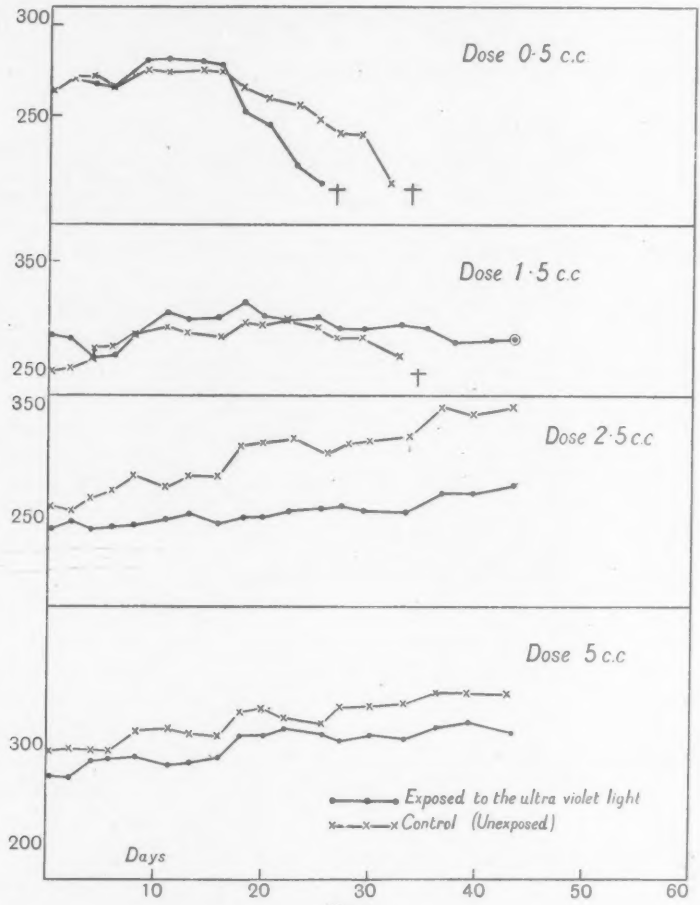

Fig. 2

os at 2 p.m. and another 2 cc. at 4.30 p.m. At 6 p.m. the same afternoon an improvement was observed. The following day the animal was normal. It was kept on a rice diet and after 10 days it developed another attack of polyneuritis. 2 cc. only were administered this time. The animal nevertheless improved and kept up its condition for 6 days.

The above experiment shows that the exposure to the ultra-violet rays did not entirely destroy the antineuritic activity of the autolysed yeast. It was desirable to ascertain whether the antineuritic activity was at all impaired by the exposure to the light. Rats were found convenient animals for that purpose. These animals if kept on a diet free from the antineuritic do not grow and eventually die after about 6 weeks. If, however, the antineuritic is supplied after the deficiency has been enforced for several weeks normal growth is immediately resumed.

In this experiment three rats were employed. Their diet was made up as follows:

Basal mixture	10 g.
Antiscorbutic equivalent to lemon juice	5 cc.
Centrifuged butter-fat	1·8 g.
Olive oil	1·6 „

The basal mixture was made up of 75 % starch, 20 % caseinogen and 5 % salts.

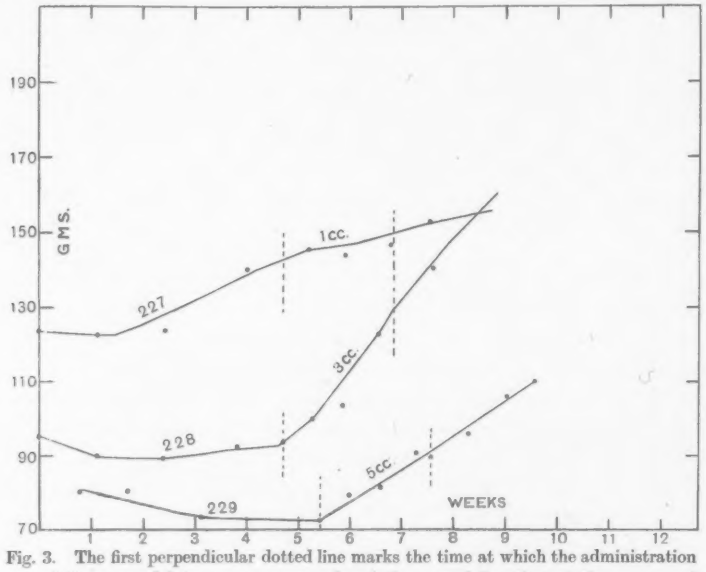

Fig. 3. The first perpendicular dotted line marks the time at which the administration of the exposed juice was commenced and the second line that of the unexposed juice of the same batch.

As will be seen from Fig. 3, two of the rats (Nos. 228 and 229) showed no growth for 4½ weeks; the third animal (No. 227) showed little growth, much below the normal. After 4½ weeks No. 227 received in its daily ration 1 cc., No. 228 3 cc. and No. 229 5 cc. of autolysed yeast juice exposed to ultra-violet rays. All the three animals commenced growing. The improvement

was least marked in rat No. 227 which received only 1 cc. of juice, the dose being evidently insufficient. After 15 days the exposed autolysed juice was replaced by unexposed autolysed juice which belonged to the same batch, and as the curves show the growth in all the three cases proceeded at the same rate as before. This experiment shows that the exposure of autolysed yeast to ultra-violet rays for 6 hours neither destroys nor vitiates to any marked degree the antineuritic activity of the juice.

The Fat-soluble A Accessory.

The influence of the ultra-violet rays on the fat-soluble *A* factor was studied next. As butter was used for the source of this accessory factor the technique had to be somewhat modified. The butter was spread in a thin layer over a glass plate which was placed in a horizontal position about 6–8 inches from the lamp. The butter was turned over several times during the exposure. The part of the plate nearest the lamp became slightly warm from the heat of the lamp but it did not reach a higher temperature than 30°. It soon became apparent that the butter was greatly altered by exposure to the ultra-violet light. It became entirely bleached, acquired an odour like that of tallow, and was unpalatable and unfit for consumption. Several preliminary experiments showed that exposure to the ultra-violet rays destroyed the fat-soluble *A* in the butter and this was confirmed on repetition. The following experiments demonstrate it. The mode of procedure adopted was the same as that employed by Drummond [1919]. Three rats were placed on a diet free from the fat-soluble *A* factor, which was made up as follows:

Basal mixture	12 g.
Antiscorbutic equivalent to lemon juice	5 cc.
Autolysed yeast juice	5 „
Olive oil	0·75 cc.

Precautions were taken to destroy any traces of the fat-soluble *A* that might be present in the basal mixture by heating the starch and the caseinogen for 3 hours at 100° C., since it has been shown by Steenbock, Boutwell and Kent [1918] and Drummond [1919] that this factor is thermolabile. The animals were kept on this diet for 18 days after which time 2 g. of the exposed butter was added to the above daily ration. This diet was continued for 25 days, when the exposed butter in the diet was replaced for two rats by ordinary butter kept for 6 hours at 35°. The butter was kept at this temperature in order to demonstrate that the slight heating effect of the lamp, which, as already mentioned, never increased the temperature to more than 30° at the nearest point, was not responsible for the destruction of the factor. On examining Fig. 4 it is seen that during the first period when the rats were receiving the diet lacking the fat-soluble *A* factor two of the animals did not grow at all, the third animal did grow. but at a rate much below the normal. The addition of the exposed butter made no difference to the growth of either

of the animals. One of the rats left on that diet developed xerophthalmia and died after about 6 weeks. When the exposed butter was replaced by unexposed butter kept for 6 hours at 35° in the diet of the remaining two rats they at once commenced growing, thus showing that the fat-soluble *A* was supplied by the addition of the unexposed but not by the exposed butter. The fat-soluble *A* factor in the butter was therefore inactivated by exposure to the ultra-violet rays.

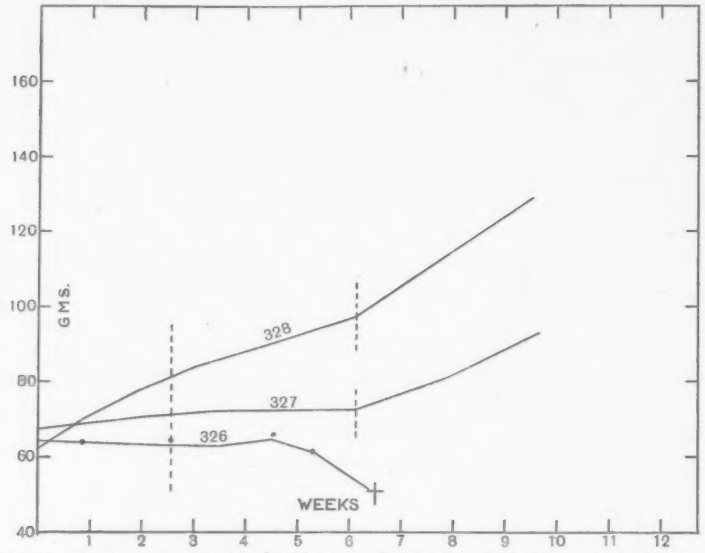

Fig. 4. The first perpendicular dotted line marks the time at which the administration of the exposed butter commenced and the second line that of the unexposed butter.

On exposing the butter to the ultra-violet rays by means of the technique described above two other factors are introduced which might be suspected to bring about the inactivation of the fat-soluble *A* factor, namely, (*a*) temperature and (*b*) ozone, which, as is well known, is produced by the action of ultra-violet light on the atmospheric oxygen. That the slight elevation of the temperature of the butter is not responsible for the inactivation is seen from the above experiment, as the addition to the diet of butter not exposed to the ultra-violet light but kept for 6 hours at 35° at once induces growth. The part played by the ozone in the inactivation of the factor, as well as the chemical changes produced by the ultra-violet light in the fat, are now being studied.

In this connection it is of interest to add that it has been previously pointed out by many investigators that the sterilisation of milk by means of ultra-violet rays imparts to the milk a peculiar taste. This is no doubt due to the alteration in the butter fat brought about by the exposure. In view of

the above observations one would also expect that the action of ultra-violet light on milk might greatly impair the activity of the fat-soluble factor in it and thus diminish its nutritive value, especially when used for the purpose of infant feeding.

SUMMARY.

1. The exposure of treated lemon juice to ultra-violet rays in neutral condition and at a H-ion concentration of P_H 2·34 for 8 hours does not influence its antiscorbutic activity.

2. The exposure of autolysed yeast juice for the same length of time does not impair its antineuritic potency.

3. Butter exposed for 8 hours to ultra-violet light undergoes a very noticeable change and the fat-soluble *A* factor in it becomes inactivated.

REFERENCES.

Drummond (1919). *Biochem. J.*, **13**, 81.
Harden and Zilva (1918). *Biochem. J.*, **12**, 259.
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.*, **36**, 577.

XX. THE INFLUENCE OF DEFICIENT NUTRITION ON THE PRODUCTION OF AGGLUTININS, COMPLEMENT AND AMBOCEPTOR.

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(*Received May 2nd, 1919.*)

At least two diseases, scurvy and beri-beri, can definitely be traced to a prolonged consumption of a faulty diet. Both experimental and clinical data fully support this view which is now accepted by the majority of scientific workers and clinicians. Other diseases designated as "deficiency diseases," like pellagra, rickets, sprue, etc., are also attributed by some to a dietary deficiency, but the scientific evidence available at present is not sufficient to warrant any definite conclusion as regards the etiology of these diseases.

As is well known scurvy and beri-beri are caused by the absence from the diet of two principles of unknown chemical constitution. By depriving the diet of certain animals of these principles pathological conditions closely resembling scurvy and beri-beri can be induced. Lately Osborne and Mendel [1912, 1] and McCollum and Davis [1913] have demonstrated the necessity of a third accessory food factor provisionally named "the fat-soluble *A* factor" for the growth and existence of the rat. This has been confirmed by other workers. The effect of this deficiency on the human subject is still uncertain, but experimental evidence is gradually accumulating which promises to reveal it.

Besides the above principles of unknown chemical constitution it has been demonstrated experimentally on various kinds of animals that the removal of some chemically known compounds and elements from the diet restricts growth in the young and makes the food inadequate for sustenance in general. These products are of inorganic and organic nature.

The indispensability of the inorganic constituents of the diet was disclosed by the early researches in nutrition. The deficiency of certain elements such as calcium or phosphorus makes itself manifest very soon in growing animals. The presence of other inorganic elements in the food also exercises a very important physiological function.

The organic portion of the diet is limited by the character of the proteins as regards their amino-acid content. Willcocks and Hopkins [1906] and

Osborne and Mendel [1912, 1, 2, 3 and 1913] have shown that certain amino-acids are indispensable for existence and cannot be synthesised by the animal organism. Consequently these must be supplied in sufficiency through the medium of the consumed food in order to ensure the well-being of the animal.

When, therefore, any of the accessory food factors or the indispensable organic or inorganic constituents are eliminated from the diet, the animals cease to grow and eventually die. In the case of deficiencies in the antineuritic and antiscorbutic factors, as already pointed out, the animals succumb to clinically recognised diseases. The pathology of the other deficiencies is not so well defined. In all cases, however, where the diet is deprived partially or completely of an essential constituent the animals under experiment soon manifest the deficiency by a retarded rate or absolute cessation of growth, by restricted propagation and by a general unhealthy appearance, so that if any vital principle of the food is withheld for a prolonged period organic changes ensue which are very marked. But besides the radical changes produced by deficient nutrition already recorded it is quite possible that decided modifications may take place in the body tissues and fluids which are not discernible macroscopically or microscopically, but which may nevertheless restrict physiologically the functions of the organism. In this connection arises the interesting problem whether the resistance to disease of the animal is in any way influenced by deficient nutrition.

There is a general belief that underfed individuals are more susceptible to infection than well fed persons and that when the former contract a disease they show less resistance and are more prone to succumb to it. But terms like "malnutrition" or "underfed" are vague expressions and lack scientific definition. There is also no definite scientific evidence even of a general character to support this belief in spite of its probability. If, however, there is a connection between imperfect nutrition and susceptibility to disease the subject becomes one of the utmost theoretical and practical importance.

Even with our present knowledge of the various foodstuffs as regards their chemical constitution and content of the accessory food factors it becomes evident on reflection that the food of some people living under modern conditions may not contain an evenly distributed and adequate amount of all the vital factors which are necessary for normal existence. It is true that, excluding abnormal conditions like war or famine, the probability of a prolonged *total* deficiency of one or more essential principles from the diet is remote, but a prolonged partial deficiency or a rigorous deficiency lasting a short period is quite possible. The effect of such an irregularly balanced diet need not necessarily manifest itself by such drastic constitutional changes as those produced by the absolute deprivation of any essential principle for a long period, but it is only reasonable to suppose that certain systemic modifications may take place. This conjecture finds some support in the observations made by Zilva and Wells [1919] who found decided histological changes in the teeth of guinea-pigs kept on a scorbutic diet at a stage when the animal

was growing normally and was apparently in normal health. One may similarly imagine that the body cells and fluids may be altered at an early stage by deficient nutrition. It becomes necessary therefore to ascertain in the first place whether the immunity of an animal is affected by a rigorous and prolonged dietetic deficiency.

Immunity is a complicated biological phenomenon and our present knowledge of the subject is a collection of certain facts supposed to be connected with it, which are, however, insufficient to explain the actual mechanism involved. Immunity, therefore, is not very amenable to quantitative estimation. That it is a variable function is evident from empirical observations. Certain phenomena, however, like phagocytosis, complement fixation, agglutination, etc., connected with immunity can be estimated quantitatively for comparative purposes, and it is therefore possible to investigate the influence of diet on such phenomena.

So far there seems to be no record of any experimental work on this subject. It has been asserted by some observers that vegetarian races are less resistant to disease than races which eat meat. Even if that assertion be correct other differential conditions under which these races live would prevent one from ascribing the lower resistance of vegetarian races to their diet. Also the resistance of carnivorous animals towards certain diseases to which herbivorous animals are subject and *vice versa* cannot be entirely explained by the differential diet owing to the number of other factors concerned.

In studying the influence of diet on immunity one has to deal with a multitude of phenomena which offer a great variety of problems to be settled. As already mentioned a diet may be deficient by lacking one of the vital chemically known or chemically unknown principles, but the deficiency may also be of a multiple character. The conceivable changes in the factors connected with immunity which may be produced in the animal organism by any deficient diet are also numerous. The problem, therefore, is very complex and its investigation difficult.

In dealing with immunity one has to consider natural immunity and acquired immunity. The former is the inherent capacity of the animal to resist infection and the latter the immunity resulting from accidental or artificial infection previously sustained. The experimental study of natural immunity would have to be carried out by injecting standardised doses of a suitable lethal organism into animals fed on the various diets to be investigated and recording the relative mortality and time taken by the animals to succumb. In order to draw definite conclusions such experiments would require a great number of animals in each set. The evident drawback of such a plan of investigation is the amount of labour necessary for the preparation of purified diets for the great number of animals a systematic inquiry of such a nature would require. The task becomes almost impossible for a single experimenter to carry out. In investigating acquired immunity one is again confronted with the difficulty of choosing from the great number of factors

which undergo a change during artificial or accidental immunisation those most suitable for study.

In this investigation the study of the influence of deficient diets on the amboceptor and agglutinin formation and the complement content has been undertaken. Owing to the complexity of the subject the experiments carried out can only be considered of a more or less preliminary character. The results obtained although of an unexpected nature are nevertheless of general interest and suggest the necessity of further investigation.

The influence of the following dietetic deficiencies on the production of the above-mentioned antibodies was studied.

(*a*) The deficiency of each of the following elements Ca, Fe, K, Cl, P, and Na in the inorganic content of the diet.

(*b*) The deficiency of certain amino-acids.

(*c*) The deficiency of the antiscorbutic, antineuritic, and the fat-soluble *A* accessory factors.

The influence of a quantitatively restricted diet on the complement content was also investigated.

Experimental.

Rats were chosen for the investigation of the action of deficient proteins, restricted mineral salts and accessory food factors on the production of amboceptor and agglutinins. These animals are very suitable for the purpose as they readily consume the experimental diet. For the study of the influence of quantitatively restricted diet on the complement content of the blood and the effect of a scorbutically restricted diet on the production of amboceptor and agglutinins guinea-pigs were employed, because these animals are well adapted for complement production and are very susceptible to scurvy. In both these instances their ordinary basal diet of oats and bran was so adjusted that the requisite deficiencies were effected without making their food unpalatable. The rats employed were mostly of the albino variety and were specially selected either from stock bred in this laboratory or from healthy stock bought outside. The animals were kept separately in wooden boxes with perforated zinc tops. The bottoms of the boxes, except in some experiments to be mentioned later, were covered with a thin layer of sawdust. The diets were delivered daily in the form of weighed balls of suitable consistency. These were weighed back the following morning to ascertain the amount eaten. The drinking water which was kept in glass dishes was changed twice a day. All the animals were closely watched during the experiment, especially for scabies, and were weighed on the average about twice a week.

The guinea-pigs too were chosen from healthy stock, great care being taken to ascertain that they had not previously been used for any inoculation experiments. These animals were kept separately in wire cages and received daily measured quantities of their respective foods in clean glass dishes. The food remnants were measured back the following day.

The rats' rations were made up by thoroughly mixing all the constituents of the diet by means of a mechanical mortar. The mixtures were brought up to a convenient consistency by the addition of water and olive oil. The diets were made up twice weekly and stored in a refrigerating room until they were wanted so that no ration was older than 3 or 4 days.

The basal ingredients of the rats' food consisted of caseinogen, except in a few cases in which that protein was not compatible with the object of the experiment, starch and a mixture of mineral salts.

The caseinogen was obtained from a commercial firm. It was pale yellow and of light texture. For experiments on the accessory food factors it was previously purified. The purification consisted of shaking the caseinogen twice with ether and once with 95 % alcohol for several hours and filtering and drying. By testing the purified caseinogen on rats it was found to be free from the antineuritic and the fat-soluble *A* accessory factors.

Pure wheaten starch was employed. It was not found necessary to purify this ingredient as it was free from both the antineuritic and the fat-soluble *A* accessory factors.

The salt mixture was made up of pure reagents in the same proportion as that used by McCollum and his colleagues, and was constituted as follows:

NaCl	1·73 g.	$CaH_4(PO_4)_2 2H_2O$	5·4
$MgSO_4$ (anhydr.) ...	2·66	Calcium lactate	13·0
$NaH_2(PO_4)H_2O$ ...	3·47	Ferric citrate	1·18
K_2HPO_4 ...	9·54		

The fat-soluble *A* factor was supplied in the form of purified butter fat. Pure butter was melted at 40° and centrifuged. The supernatant layer of fat was then carefully syphoned off leaving behind the caseinogen, water and impurities.

Autolysed yeast was added to the diet to supply the antineuritic. Ordinary top fermentation brewer's yeast was pressed out in order to remove the adhering wort. It was then introduced into flasks plugged with cotton wool and placed in the hot room at 37° until it had liquefied. The autolysed mixture was then filtered through a Buchner funnel by means of the suction pump. The resulting filtrate was brown and fairly transparent, and was rich in the antineuritic factor. As the autolysed yeast solution contains a good deal of the inorganic constituents of the yeast it was necessary to treat it further when used for the experiments in which the inorganic constituents of the diet were limited. This treatment will be described later.

The antiscorbutic factor was introduced into the diet in the form of lemon juice treated according to the method described by Harden and Zilva [1918]. This solution has the advantage of containing almost all the antiscorbutic content of the lemon juice associated with little extraneous matter and can be used in very concentrated form if necessary.

In studying the production of the antibodies the animals were fed on the restricted diet for several weeks, after which time they were immunised by

three injections of increased doses of organisms with an interval of 7 days between each injection. The animals were bled out a week after the last injection. The blood was then allowed to clot and centrifuged and the supernatant serum employed for the estimation of the agglutinin and amboceptor.

The immunisation was carried out with the typhoid organism "Simpson." Young 24-hour cultures were emulsified for the purpose and the emulsion brought up to a dilution of 1000 million per cc. It was then killed by heating it at 60° for ½ hour. The same batch of emulsion which was kept in a refrigerating room was used for each group of experiments. The animals were weighed on the day of the injection and their doses were calculated in proportion to their weight. All the doses were brought up in volume with saline to 1 cc., and were injected subcutaneously in the abdominal region.

In estimating the agglutinins a "Simpson" typhoid emulsion containing 2000 million organisms per cc. was used. The different dilutions of the sera and the emulsion were kept at a temperature of 37° for 1 hour and read off 15 minutes after and again the following day.

The amboceptor was estimated by means of the complement fixation. For that purpose ½ cc. of each dilution of the serum plus ½ cc. of the "Simpson" emulsion of the same strength as that used for the agglutination plus ½ cc. of complement were kept for one hour at 37°, after which time to each tube was added ½ cc. of washed sheep's blood corpuscles and ½ cc. of haemolytic amboceptor. The tubes were then kept for another hour at 37° and removed to the refrigerating room until the following morning, when the readings were taken. With each set two control tubes containing 1 in 25 serum without emulsion and a double quantity of emulsion, namely 1 cc., without serum were also set up. On each occasion a stock antityphoid serum was titred out for agglutinins and complement fixing bodies to demonstrate the activity of the reagents employed.

For the complement fresh guinea-pig serum diluted $\frac{1}{10}$ with saline was used. The haemolytic amboceptor was titrated the day of the estimation and double the registered strength necessary for haemolysis was employed.

Before proceeding with the experiments it was necessary to show that by injecting normal guinea-pigs and rats with the "Simpson" typhoid emulsion the production of agglutinins and amboceptor could be effected. It was also necessary to ascertain the number of injections required for the production of suitable quantities of agglutinins and amboceptor normally so that a restricted production of these antibodies could be demonstrated by comparison. For that purpose the following experiment was carried out.

Rat No. 55, weighing 54 g., received an injection of 1 cc. containing 40 million "Simpson" typhoid organisms, and was bled out a week after the injection.

Rat No. 56, weighing 46 g., received an injection of 1 cc. containing 40 million organisms and a week after another cc. containing 80 million organisms and was bled out a week after the second injection.

Guinea-pig No. 313, weighing 402 g., received an injection of 1 cc. containing 250 million organisms, and was bled out a week after the injection.

Guinea-pig No. 314, weighing 383 g., received an injection of 1 cc. containing 250 million organisms and another injection of 1 cc. a week after containing 500 million organisms. A week after the second injection this animal was bled out. Table I gives the agglutination and amboceptor titres of the above animals.

Table I.

In this and all the other tables + indicates agglutination or lysis.

Agglutination.

	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
Rat 55 (one injection)	+++	+++	+++	++	+	–	–	–
Rat 56 (two injections)	+++	+++	+++	+++	+++	++	++	–
Guinea-pig No. 313 (one injection)	+++	+++	+++	++	+	+	–	–
Guinea-pig No. 314 (two injections)	+++	+++	+++	+++	+++	++	+	–
Stock antityphoid serum	+++	+++	+++	+++	+++	+++	+++	++

Amboceptor.

	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Rat 55 (one injection)	+++	+++	+++	+++	+++	+++	+++
Rat 56 (two injections)	–	–	+++	+++	+++	+++	+++
Guinea-pig No. 313 (one injection)	+++	+++	+++	+++	+++	+++	+++
Guinea-pig No. 314 (two injections)	–	–	+	+++	+++	+++	+++
Stock antityphoid	–	–	–	–	–	+	++

It is seen from the above table that both in the normal guinea-pig and rat a very marked production of agglutinins was achieved with two injections. The amboceptor production did not respond so well but a decided change in the titre was effected with two injections. It was, therefore, decided to submit the experimental animals in this investigation to three injections with an interval of one week. The strength of the doses was to be calculated according to the body weight and was to be as follows: first injection 1 million organisms per g. body weight, second injection 2 million organisms per g. body weight, and third injection 4 million organisms per g. body weight. Lower dilutions of the serums, namely $\frac{1}{5}$ and $\frac{1}{10}$, were also to be titrated for the amboceptors.

As all the deficiency diets investigated could not conveniently be tested together, the experiments were divided into various sets. Several animals were put on each diet and their blood was pooled in order to obviate the error of idiosyncracy. To make conditions as nearly as possible equal the weights, sex and colour of the animals of each set were kept the same. Each

set had a group of rats on a control diet which was theoretically complete and the daily ration consisted of the following ingredients:

Basal mixture	10·6 g.
Purified butter	1·7 ,,
Autolysed yeast	5 cc.
Olive oil	0·25 ,,
Antiscorbutic equivalent to lemon juice	5 ,,

The basal mixture was made up of 75 % of starch, 20 % of caseinogen and 5 % of salts.

The above diet contains all the known accessory factors. For the sake of convenience, the fat-soluble *A*, the antineuritic and the antiscorbutic factors will be referred to in this communication as *A*, *B* and *C* respectively and the theoretically complete control diet as the *ABC* diet.

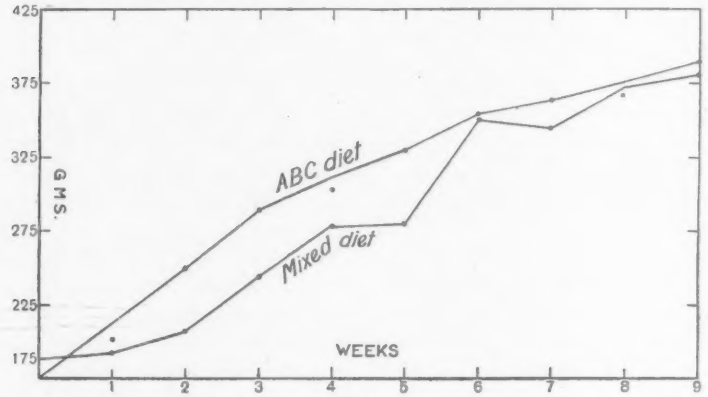

Fig. 1.

The first thing to ascertain was how rats fed on an *ABC* diet respond to injections in agglutinin and amboceptor formation in comparison with rats fed on an ordinary mixed diet. Two groups of rats each containing 3 animals were employed in this experiment. One group received an *ABC* diet, the other was fed on a mixed diet consisting of cabbage, boiled potatoes, scraps of bone and meat, biscuit meal, bread, carrots and boiled rice. After having subsisted for 6 weeks on the above diets the animals were immunised and bled out 9 weeks after the commencement of the experiment. The group receiving the *ABC* diet yielded 8 cc., the other group 9 cc. of blood. As will be seen from Fig. 1, which represents the growth curves of the two groups, the animals in both groups gained well in weight and the final weights were about the same in both cases. The increase in weight of the mixed diet group, however, was more erratic than that of the *ABC* group, which was most probably due to the lack of well-balanced uniformity in the diet of the former group, the

animals of which were left to choose their food from the offered mixture by instinct.

Table II gives the agglutination and amboceptor titres of the two groups. In both groups there was a very marked production of agglutinins and amboceptor, especially of the former, so that any differential production of these antibodies could not be missed. There is, however, no marked differentiation to be observed in the production of either of the antibodies. There is a slight difference in the amboceptor titres of the two groups, but it is of an order that may be neglected.

Table II.

	Agglutination.			*Amboceptor.*							
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
ABC purified diet	+++	+	–	–	–	±	++	++±	+++	+++	+++
Mixed diet	+++	+	–	–	+	++	+++	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	+++	–	–	–	–	–	–	+	++

Having ascertained that rats fed on an *ABC* diet respond to injections in the formation of agglutinins and amboceptor under the conditions described equally well as rats fed on a good mixed diet, it was possible to employ this diet as a control in the study of the various deficient diets.

It was previously mentioned that it has been definitely established that the inorganic constituents of the food are indispensable for existence. Lately Osborne and Mendel [1918] made a detailed study of the influence of various inorganic elements in nutrition and found that calcium and phosphorus were the only elements which when singly reduced to very low limits had a retarding effect on the growth of rats. In the present investigation the study of the influence of a deficiency of iron, calcium, potassium, phosphorus, chlorine and sodium on the production of the immune bodies was attempted. For this purpose in devising the diets precautions had to be taken in order to reduce the presence of the respective elements to a minimum. This was accomplished in all the cases with the exception of the sodium and chlorine, in which owing to an oversight the amounts of those elements consumed were rather higher than was intended. In all the groups wheaten starch was used as it was found to be fairly free from the inorganic constituents under investigation. Caseinogen was employed for the protein portion of the diet with the exception of the group which received a phosphorus-free diet, in which case egg-white was used instead. The egg-white was obtained from a commercial firm and did not contain any phosphorus.

The autolysed yeast could not be used in the ordinary way to supply the antineuritic factor as it contained a good proportion of the inorganic constituents of the yeast. It was, therefore, treated in a manner which reduced its salt content to a minimum. The procedure was as follows. Five parts by volume of autolysed yeast were mixed with 95 parts of absolute alcohol and

well stirred. A viscous precipitate containing mostly proteins and their degradation products and salts was formed. This was filtered off yielding a clear yellow-coloured filtrate. The alcoholic filtrate was then evaporated to dryness *in vacuo* at 40° and the residue obtained dissolved in a volume of distilled water equivalent to the original juice. This solution was tested on a polyneuritic pigeon and was found to be active. 3 cc. of this active solution was given in the daily ration and was sufficient to keep a control group of rats in good health. The treated lemon juice which was used to supply the anti-scorbutic factor in the diet contained so little inorganic matter that it could be neglected. The purified butter with the exception of the sodium chloride adhering to it contained little inorganic matter. Unfortunately the sodium chloride supplied by this source, although very low, was overlooked and, as has already been mentioned, made the diets intended to be deficient in these elements contain rather significant traces of sodium and chlorine. The inorganic constituents of the various diets were made up as follows:

Table III.

	Diet low in Iron	Diet low in Calcium	Diet low in Potassium
	g.	g.	g.
NaCl	4·6	4·6	4·6
$MgSO_4$ (anhydrous)	7·4	7·4	7·4
NaH_2PO_4 ...	9·4	9·4	9·4
K_2HPO_4	25·8	25·8	—
$CaH_2(PO_4)_2$ ...	14·6	—	14·6
Ca lactate ...	35·3	—	35·3
Ferric citrate ...	—	3·2	3·2

All the above were made up with starch to 100 g.

Diet low in Sodium.

KCl	6·0	$CaH_2(PO_4)_2$ ...	14·6
$MgSO_4$ (anhydrous)	7·4	Ca lactate	35·3
K_2HPO_4	25·8	Ferric citrate ...	3·2

Made up to 100 g. with starch.

Diet low in Chlorine.

$MgSO_4$ (anhydrous)	7·4	$CaH_2(PO_4)_2$ ...	14·6
NaH_2PO_4	9·4	Ca lactate	35·3
K_2HPO_4	25·8	Ferric citrate ...	3·2

Made up to 100 g. with starch.

Diet low in Phosphorus.

NaCl	4·6	$CaCO_3$	6·3
$MgSO_4$ (anhydrous)	7·4	Na_2CO_3	4·2
K_2CO_3	20·0	Ferric citrate ...	3·2
Ca lactate	35·3		

Made up to 100 g. with starch.

The basal mixtures for these experiments consisted of 80 % of starch, 20 % of protein and 5 % of the respective salt mixtures. The diets were made up in the same proportions as the *ABC* diet with the exception that only 3 cc. of the treated autolysed yeast was given.

Samples of the diets were analysed for the various elements with the following results:

Diet low in iron	Absent
„ „ calcium ...	0·014 %
„ „ potassium ...	Undeterminable traces
„ „ sodium ...	0·014 % calculated from Cl
„ „ chlorine ...	0·021 %
„ „ phosphorus	0·05 %

All the rats in this experiment received distilled water to drink and were housed in boxes containing perforated zinc floors. It was not advisable to use sawdust in this instance as some of it might have been consumed with the food and thus formed a source for inorganic constituents.

For convenience these experiments were divided into two sets. In the first set the deficiency of iron, calcium and potassium was investigated. In the second set the deficiency of sodium, chlorine and phosphorus was studied. Each set had a control group of animals. Each group of the first set consisted of five rats. These animals were kept 85 days on the experimental diets. The group on diet low in Ca yielded 16 cc., in K 20·5 cc., in Fe 21·5 cc. and the control 19 cc. of blood. Fig. 2 shows that the animals receiving the diets deficient in Fe and K grew almost as well as the control group of rats, although the K group showed a slight decline towards the end of the experiment. On the other hand, the group deficient in Ca showed a decided restriction in their development. By examining Table IV it will be seen, however, that the agglutinins and amboceptor titres are almost identical in all the groups.

The second set of animals had 3 rats in each group. It will be seen from Fig. 3, that although the groups deficient in sodium and chlorine developed as well as the control in this set, the group receiving the diet low in phosphorus not only failed to show any growth but actually declined. In order to obviate the loss of the animals in this group the experiment was terminated after 54 days. The following quantities of blood were obtained. The sodium group yielded 9 cc., the chlorine 10 cc., the phosphorus 9 cc. and the control 9 cc. of blood. It is to be noticed that the phosphorus group although the lightest in weight yielded as much blood as the other groups. By examining Table V it is seen that no differentiation in the agglutinins and amboceptor formation in the groups which received the diets deficient in Na and Cl could be observed. The animals which were fed on the diet low in phosphorus showed a decidedly lower titre in the agglutinins and amboceptor. In fact hardly any amboceptor formation took place in this group. As will be seen from the other experiments which follow, this was the only group that showed a lower amboceptor titre.

In arranging the above diets it was impossible to eliminate from the basal

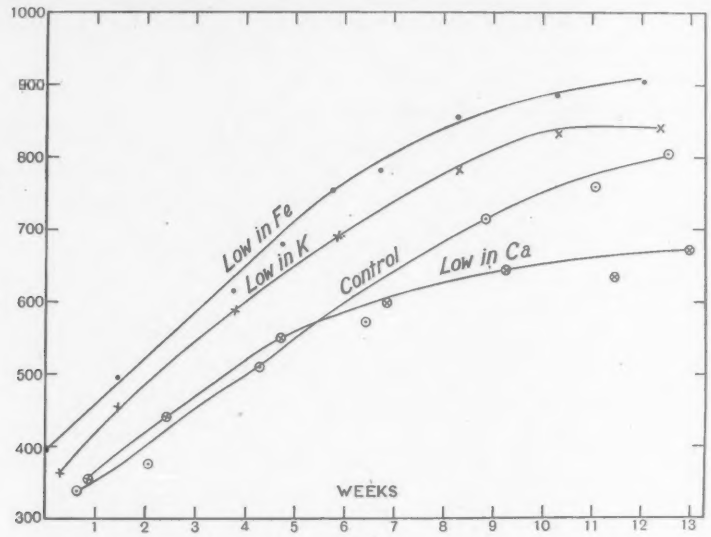
1000
900
800
700
600
500
400
300
Low in Fe
Low in K
Control
Low in Ca
WEEKS
1
2
3
4
5
6
7
8
9
10
11
12
13

Fig. 2

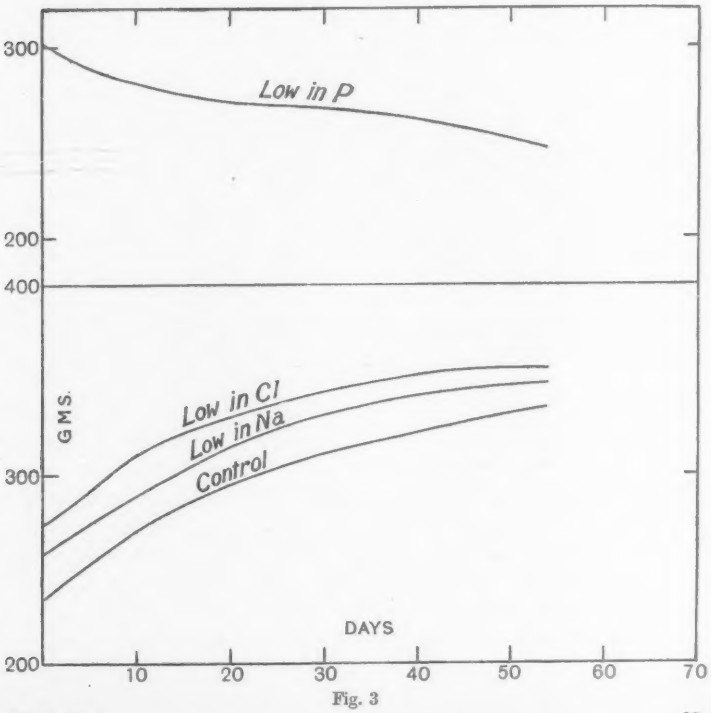
300
Low in P
200
400
GMS.
Low in Cl
Low in Na
Control
300
DAYS
200
10
20
30
40
50
60
70

Fig. 3

salt mixture any one element without at the same time altering quantitatively the content of some of the other constituents. This alteration was not of an important character as care was taken not to establish a second deficiency, and in view of the results obtained it can be ignored.

Table IV.

	Agglutination.					*Amboceptor.*				
	1/800	1/1600	1/3200	1/6400	1/25	1/50	1/100	1/200	1/400	1/800
Control diet	+++	+++	+++	+	–	++	+++	+++	+++	+++
Ca-free diet	+++	+++	+++	+++	–	+	+++	+++	+++	+++
K-free diet	+++	++	–	–	–	+½	+++	+++	+++	+++
Fe-free diet	+++	+++	++	–	–	++½	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	–	–	–	–	++	+++

Table V.

Agglutination.

	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800
Control diet	+++	+++	+++	++	++	++	+
Diet low in phosphorus	+++	++	+	trace	–	–	–
Diet low in sodium ...	+++	+++	+++	+++	++	+	–
Diet low in chlorine ...	+++	+++	+++	+++	++	+	–
Stock antityphoid ... serum	+++	+++	+++	+++	++	++	+

Amboceptor.

	1/5	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Control diet	½	+½	++	+++½	trace +++	+++	+++	+++	+++
Diet low in phosphorus	trace +++	trace +++	+++	+++	+++	+++	+++	+++	+++
Diet low in sodium	–	–	½	+	trace +++	+++	+++	+++	+++
Diet low in chlorine	–	–	½	trace +++	+++	+++	+++	+++	+++
Stock antity-phoid serum	–	–	–	–	–	+	+½	++	+++

On examining the composition of the diet low in phosphorus it is seen that two other factors were also altered. The salt mixture was slightly more alkaline owing to the higher content of the carbonates of sodium and potassium which replaced the phosphates of those salts, and the caseinogen was replaced by egg-white, which from observations made in control experiments does not seem to be as good a source of protein as caseinogen. As the result obtained with this group is interesting the subject will have to be studied further before definite conclusions can be arrived at.

The next thing to be investigated was the influence of diets deficient in certain amino-acids on the production of the antibodies in question. Two sets

of experiments were devoted to this subject. In the first set a low intake of caseinogen was studied, in the second the action of gliadin as the sole source of protein in the diet.

Osborne and Mendel [1915] were the first to demonstrate that if caseinogen formed 18 % of a diet it was adequate to ensure normal growth in rats; if, however, the content of that protein in the diet was reduced to 9 % the rate of growth of the animals was greatly impaired. The explanation was advanced that cystine was the limiting factor, caseinogen having a low cystine content. When it forms as much as 18 % of the diet the amount of cystine supplied through its medium is sufficient to satisfy the requirements of the animals, when, however, it forms only 9 % of the diet the amount of cystine consumed becomes deficient and manifests itself by the restricted growth of the animals. This explanation was confirmed by the fact that the addition of cystine to a low caseinogen diet raised its nutritive value to the same level as that of lactalbumin.

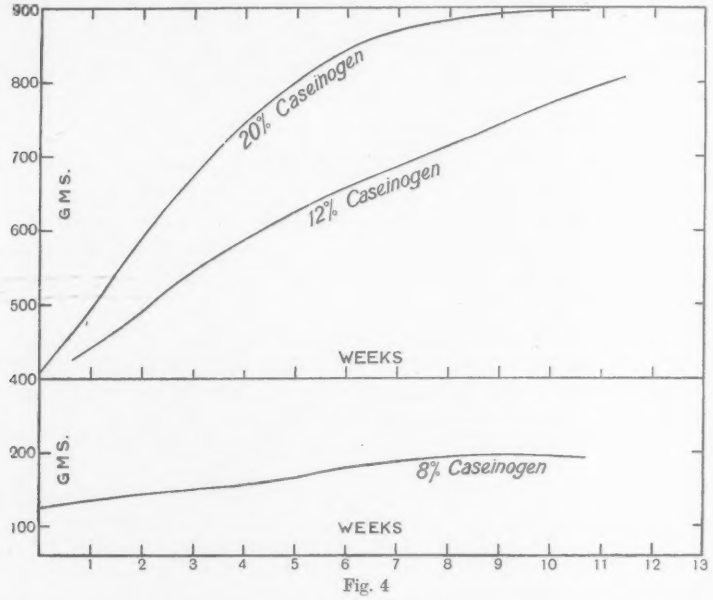

Fig. 4

In order to study the deficiency of cystine, diets of low caseinogen content were used in this investigation. Three groups of rats receiving 20 %, 12 % and 8 % respectively, were used. With the exception of the varied quantities of the caseinogen and starch the diets were in other respects similar to the *ABC* diets. Six animals were started in each group, but only two survived in the group receiving a diet with 8 % of caseinogen. Fig. 4 gives the weight curves of the three groups. It is seen that the group which received the 8 %

caseinogen diet did not grow at all. The group fed on the 12 % caseinogen diet grew but did not do as well as those on the 20 % diet. When killed 23 cc. of blood was collected from the group of six rats fed on the 20 % caseinogen diet, 15 cc. from the group of six rats which received 12 % diet, and $4\frac{1}{2}$ cc. from the group of two rats which survived on the 8 % diet. Table VI shows that no marked difference can be observed either in the agglutinins or in the amboceptor formation of the three groups in spite of the restricted growth which the groups receiving 12 % and 8 % of caseinogen manifested.

Table VI.

	Agglutination.				*Amboceptor.*			
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200
Diet containing 20 % caseinogen	+++	+++	++	–	±	+++	+++	+++
Diet containing 12 % caseinogen	+++	+++	–	–	±	+++	+++	+++
Diet containing 8 % caseinogen	+++	++	–	–	++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	–	–	+	++	+++

According to Osborne and Mendel [1914, 1916], gliadin as the sole protein in a diet suffices to maintain rats, but is insufficient to promote growth in young animals unless it is supplemented by lysine, an amino-acid which this protein does not contain. The influence of gliadin was studied in this investigation on a set of rats containing one group receiving an *ABC* diet with caseinogen as the protein and another group having the caseinogen replaced by gliadin.

The gliadin was prepared from wheat flour by the method described by Osborne [1910]. The product was precipitated twice with water and once with absolute alcohol and was thus carefully purified. The basal mixtures in these experiments were made up as follows:

Control diet.

Starch 77 %, Caseinogen 18 %, Salts 5 %.

Gliadin diet.

Starch 77 %, Gliadin 18 %, Salts 5 %.

The above basal mixtures were mixed with the necessary ingredients in proportion already described to form an *ABC* diet.

The weight curves of the two groups (Fig. 5) show that rats which were fed on the gliadin diet failed to grow, thus confirming the observations of Osborne and Mendel. Six animals were started in each group, but only four survived in the gliadin group and consequently the blood of four animals in each of the above groups was pooled for examination. These rats were immunised after 38 days instead of 6 weeks as it was feared that more animals

in the gliadin group might die if the experiment were prolonged. The animals in the gliadin group yielded 9 cc. of blood, the four animals in the control group yielded 16 cc. of blood. Table VII shows that the gliadin diet made no difference to the agglutinin and amboceptor production, although the animals in this group showed no growth owing to the restriction in the diet. The view that the nutritive restriction of gliadin is due to the lack of lysine is not altogether supported by the observations of McCollum, Simmonds and Pitz [1917] and Geiling [1917]. The former found that when maize kernel, which is poor in lysine, is supplemented by gelatin, which is very rich in that amino-acid, the nutritive value of the maize is not enhanced. The latter is led to believe by his investigation that lysine is not indispensable for existence of the mouse. Whatever the deficiency of gliadin may be due to it does not inhibit the production of agglutinins and amboceptor.

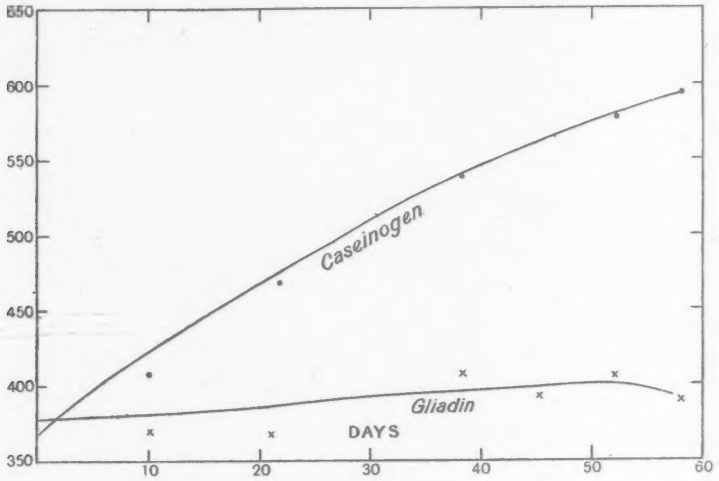

Fig. 5

Table VII.

	Agglutination.				*Amboceptor.*				
	1/800	1/1600	1/3200	1/6400	1/100	1/200	1/400	1/800	1/1600
Control diet containing 18 % caseinogen	+++	+	–	–	–	+	+++	+++	+++
Diet containing 18 % gliadin	+++	++	–	–	–	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	++	–	–	–	+	++	+++

The following sets of experiments were devoted to the investigation of the influence of the lack of the accessory factors. Observations were carried out on animals subsisting on diets deficient in the antineuritic, fat-soluble *A*, and antiscorbutic factors. McCollum and Davies have revealed by their researches that rats lacking the antineuritic factor in their diet fail to grow and die after about 6 weeks, often manifesting decided neuritic lesions. This was confirmed by Drummond [1917] and by Harden and Zilva [1918, 2]. If these animals are deprived of the fat-soluble *A* factor they may show a certain amount of growth during the initial stage of the experiment, but eventually also cease to develop and die. In most of these cases the animals display a condition of the eyes resembling xerophthalmia. Although rats do not actually manifest scorbutic lesions when kept on a scorbutic diet there seems according to Harden and Zilva [1918, 2] and Drummond [1919] to be evidence that they do not thrive as well on such diets as they do when the antiscorbutic factor is supplied. The standard *ABC* diet used in these experiments contained all the three factors. The *AB* diet was deprived of the treated lemon juice, the *BC* diet of the butter fat, and the *AC* diet of the autolysed yeast. They were made up as follows:

AB diet daily ration.

Basal mixture	10 g.	Autolysed yeast	5 c.c.
Purified butter	1·85 g.	Olive oil	2·5 c.c.

BC diet daily ration.

Basal mixture	12 g.	Antiscorbutic, equivalent to lemon juice...	5 c.c.
Autolysed yeast	5 c.c.	Olive oil	0·75 c.c.

AC diet daily ration.

Basal mixture	10 g.	Centrifuged butter fat ...	1·8 g.
Antiscorbutic, equivalent to lemon juice	5 c.c.	Olive oil	1·6 g.

The basal mixtures in all the above diets were of the same composition as the control *ABC* diet already described.

As these experiments were intended to last 9 weeks the majority of the animals subsisting on the *BC* and *AC* diets would by that time have succumbed to the deficiency and thus frustrated the object of the experiment. It was, therefore, found necessary to add at times small quantities of butter and autolysed yeast to the respective groups in order to prolong the existence of the animals, but the quantities of these addenda had to be of an order insufficient to promote normal growth. It will be seen from Fig. 6 that the *ABC* group grew best, the *AB* group did almost as well, while the *BC* group and more so the *AC* group show restricted development. Both these groups exhibited the characteristic appearance of animals existing on restricted diets. Five rats were used in each group of this experiment. By examining Table VIII it is seen that there was no change in the amboceptor production, but the

agglutinin titres showed a great variation, none of the groups receiving the deficient diets producing as much as the control group. Taking this as an indication that the absence of the accessory factors from the diets influences the agglutinin production in the animals it became desirable to repeat the experiment. A set of animals, therefore, similar to the above were again submitted to the same treatment as the first, but as will be seen from Table IX no differentiation in the agglutinin titres was obtained the second time. This would suggest that the impaired agglutinin formation could not be ascribed to the consumption of the deficient diets. Another experiment about to be described gives further support to this view.

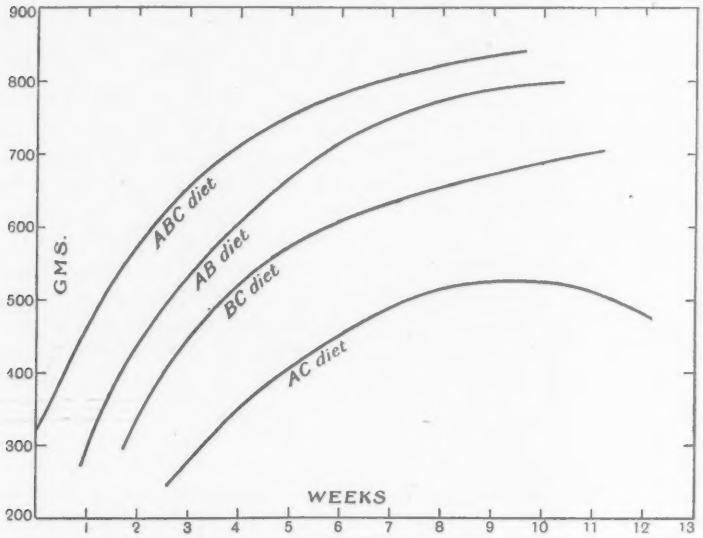

Fig. 6

Table VIII.

	Agglutination.						*Amboceptor.*							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
ABC purified diet	+++	+++	+++	+++	+	–	–	++	+++	+++	+++	+++	+++	+++
AB purified diet	+++	+++	+	–	–	–	–	–	++	+++	+++	+++	+++	+++
BC purified diet	+++	+++	+	–	–	–	–	–	+	++	+++	+++	+++	+++
AC purified diet	+++	+	–	–	–	–	–	+++	+++	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–

Table IX.

	Agglutination.		*Amboceptor.*							
	1/3200	1/6400	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
ABC purified diet	+++	–	–	–	++	++±	+++	+++	+++	+++
AB purified diet	+++	+	–	–	++	++±	+++	++±	+++	+++
BC purified diet	+++	–	–	±	++±	+++	+++	+++	+++	+++
AC purified diet	+++	++	–	–	+	++±	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	–	–	–	–	±	+	++	+++

It has already been mentioned that rats kept on a scorbutic diet do not show scorbutic lesions. It was, therefore, of interest to study the influence of the antiscorbutic deficiency on the production of the antibodies in guinea-pigs, which are very susceptible to scurvy. These animals, if kept on a scorbutic diet, manifest signs of scurvy after about 9 days and succumb to the disease about a month after. After about 15 days these animals usually cease to grow, and eventually decline in weight. It is possible by administration of small doses of antiscorbutics just to maintain the weights of the guinea-pigs and keep the animals alive in this way for months. Animals in such a condition are decidedly scorbutic, as can be proved by chloroforming them and holding a post mortem examination. By taking advantage of this fact it was possible to investigate the subject on guinea-pigs. Three groups of guinea-pigs were employed in this experiment. One group received a basal diet of oats and bran and 40 cc. of autoclaved milk and occasional doses of orange juice given *per os* at intervals sufficient to maintain the weight of the animals constant. The second group received a quantitatively restricted mixed diet containing plenty of antiscorbutics. The food was given in quantities sufficient to maintain the weights of the animals, but insufficient to permit growth. It consisted of a daily ration of 5 g. of cabbage, 10–20 g. of bran and 30 cc. of fresh milk containing 10 cc. of treated lemon juice. The third group received oats, bran, milk, cabbage and roots *ad lib.* All the animals were kept 73 days on their diets. The animals received 250 million organisms in the first, 500 million in the second, and 1000 million in the third injection per 400 g. of body weight. Fig. 7 shows the weight curves of the three groups. Four animals were bled out in each group and the unrestricted mixed diet group yielded 48 cc., the restricted mixed diet group 31 cc. and the scorbutic group 26 cc. All the animals of the scorbutic group showed well-marked signs of scurvy, whereas all the animals of the other groups were entirely free from any trace of scorbutic lesions. The agglutination and amboceptor titres, which are represented in Table X, do not show any differentiation in the three groups. These results go further to support the view that the impaired agglutinin production observed

in the first set of rats fed on the diets deficient in the accessory factors was not due to the diet.

It is well known that guinea-pigs form a good source of complement and it was of interest to ascertain whether, if these animals are restricted in their diet, the complement activity of their blood is impaired. The blood of the animals of the three groups described above was therefore tested for its complement content. Varying quantities of the serums were added to tubes containing a haemolytic amboceptor and washed blood corpuscles. As Table XI discloses, no variations in the titre could be observed, thus showing that the bloods of the three groups were of almost the same activity.

Table X.

	Agglutination					*Amboceptor*							
	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Scorbutic diet	+++	+	–	–	–	–	+	++	+++	+++	+++	+++	+++
Restricted mixed diet	+++	++	–	–	–	–	+	++	+++	+++	+++	+++	+++
Unrestricted mixed diet	+++	++	–	–	–	–	–	+	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–

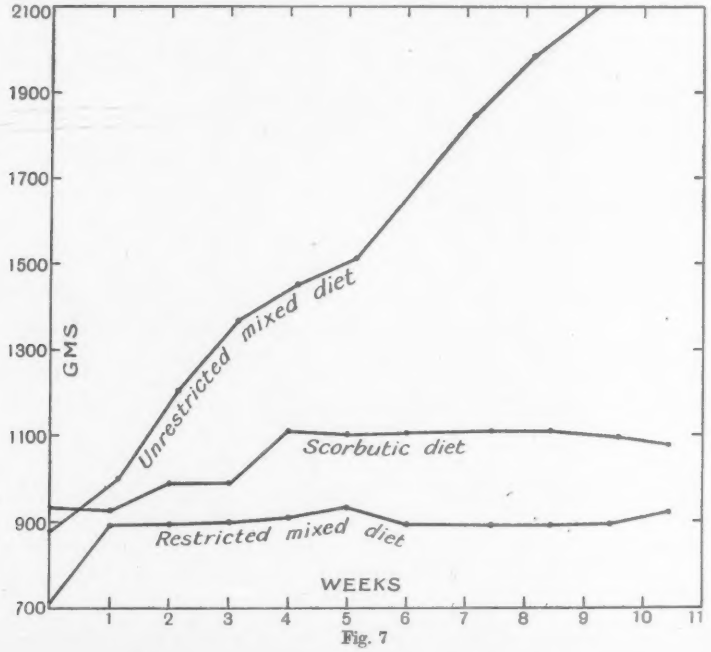

Fig. 7

Table XI.

Complement $\frac{1}{4}$	0·2 c.c.	0·1 c.c.	0·05 c.c.
Scorbutic diet	+++	++	–
Restricted mixed diet ...	+++	++	–
Unrestricted mixed diet ...	+++	+++	–

Table XII.

Complement $\frac{1}{4}$	0·2 c.c.	0·1 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
Restricted mixed diet ...	+++	+++	++	–	–
Unrestricted mixed diet ...	+++	+++	–	–	–

Another set of experiments on the subject was instituted with the object of ascertaining whether a longer period of dietetic restriction would influence the activity of the complement.

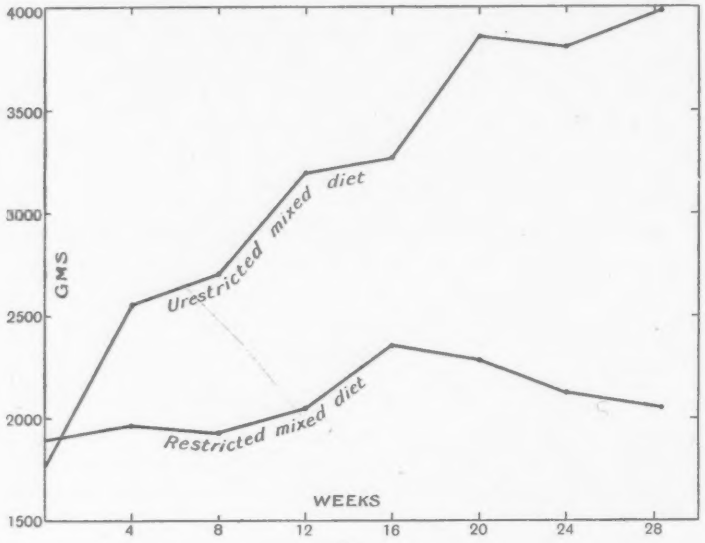

Fig. 8

Two groups of six guinea-pigs each, kept on the experimental diet for 202 days, were investigated. One group received a quantitatively restricted mixed diet and was of the same nature as the one described above. The other group received a mixed diet *ad lib.* Fig. 8 shows their weight curves which are similar in character as those of the previous experiment. At the end of the experiment these animals were bled out and their blood was pooled. The restricted mixed diet group yielded 67 cc. and the unrestricted mixed diet group 111 cc. of blood. As will be seen from Table XII the prolonged dietetic restriction did not influence the complement content of the blood of these animals.

Conclusion.

Of all the groups of animals tried on the various deficient diets only the group which received a diet deficient in phosphorus showed a decidedly poorer response to the inoculations in the production of amboceptor and agglutinins. One set of animals subsisting on diets deficient in the accessory food factors showed a low production of agglutinins only, but as this did not agree with the results obtained on guinea-pigs fed on a scorbutic diet this particular experiment was repeated. The results obtained on repetition failed to confirm the differential production of the agglutinins in rats fed on diets deficient in the accessory food factors. On the other hand, the majority of the groups of animals fed on deficient diets failed to show any difference in the production of both agglutinins and amboceptor. This becomes more remarkable when it is seen that some of the groups owing to the dietetic deficiency manifested very restricted growth or no growth at all and were altogether in a much poorer condition than the control animals. It is true that none of these experiments has been repeated, but we have several instances in this investigation where owing to various nutritional deficiencies the animals have displayed a very inferior condition to the control rats fed on a theoretically complete diet and yet have shown no differential production of the antibodies. The results of the groups fed on the 12 % and 8 % caseinogen diets, on the diet low in calcium and on the gliadin diet may be cited in this connection. It is, therefore, interesting that the group of animals fed on a diet low in phosphorus should be the only one showing a well marked differentiation in their agglutinins and amboceptor titres. This, of course, invites further investigation in order to establish the fact definitely. The results of the experiment can at present only be considered as an indication.

This investigation has only dealt with two antibodies produced under certain definite conditions. What actual bearing these antibodies have on immunity is not well established, but evidently they have some unexplained indirect connection with it, as the Widal's reaction seems to suggest in the case of agglutination. On the whole the subject is still very obscure. In the case of bacteriolysins it is known that as a result of infection or inoculation the corresponding bacteriolytic amboceptors are formed. This is demonstrated by bacteriolysis *in vitro* and *in vivo*; yet this bacteriolytic power of the blood of the organism does not remain, while the increased resistance to the disease is sometimes established for years. It is, therefore, plain that the actual capacity of the animal for antibody formation cannot be taken as a definite indication of the power to resist disease, although in conjunction with other observations it may give valuable information on the subject. A systematic study of the influence of nutrition on the production of immunity, although complex and laborious, is urgently called for and may not only prove to be of direct clinical value but may elucidate many obscure points in the complicated mechanism of immunity.

SUMMARY.

The influence of deficient diets on the production of agglutinins and amboceptor in rats was investigated.

The following deficiencies were studied.

1. Diets low in iron, calcium, potassium, sodium, chlorine and phosphorus.
2. Diets containing 12 % and 8 % of caseinogen as a source of protein.
3. Diets containing 18 % of gliadin as the sole source of protein.
4. Diets deficient in each of the three accessory food factors.

Although several of the deficiencies became manifest by the restricted growth and the poor condition of the animals, no differentiation in the titres of the agglutinins and amboceptor could be recorded, except in the group receiving the diet low in phosphorus. Guinea-pigs fed on an unrestricted mixed diet, quantitatively restricted mixed diet and a scorbutic diet showed no differentiation in the amboceptor and agglutinin titres, and in the complement activity of the blood.

Guinea-pigs kept on an unrestricted mixed diet and a quantitatively restricted diet for about 6 months showed no differentiation in the complement activity of the blood.

In conclusion I wish to express my gratitude to Dr H. Schütze for the valuable help and advice he has given me in connection with the bacteriological work of this investigation. Dr Schütze has been good enough to check independently all the readings of the agglutinin and amboceptor titres.

REFERENCES.

Drummond (1917). *Biochem. J.*, **11**, 255.
—— (1919). *Biochem. J.*, **13**, 77.
Geiling (1917). *J. Biol. Chem.*, **31**, 173.
Harden and Zilva (1918, 1). *Biochem. J.*, **12**, 259.
—— (1918, 2). *Biochem. J.*, **12**, 408.
McCollum and Davis (1913). *J. Biol. Chem.*, **15**, 167.
McCollum, Simmonds and Pitz (1917). *J. Biol. Chem.*, **28**, 483.
Osborne (1910). *Handb. Biochem. Method. Abderhalden*, **2**, 320.
Osborne and Mendel (1912, 1). *J. Biol. Chem.*, **12**, 81.
—— (1912, 2). *Zeitsch. physiol. Chem.*, **80**, 307.
—— (1912, 3). *J. Biol. Chem.*, **13**, 473.
—— (1913). *Science*, **37**, 185.
—— (1914). *J. Biol. Chem.*, **17**, 325.
—— (1915). *J. Biol. Chem.*, **20**, 351.
—— (1916). *J. Biol. Chem.*, **25**, 1.
—— (1918). *J. Biol. Chem.*, **34**, 131.
Willcocks and Hopkins (1906). *J. Physiol.*, **35**, 88.
Zilva and Wells (1919). *Proc. Roy. Soc.*, B. **90**, 505.

XXI. THE NOMENCLATURE OF BLOOD PIGMENT AND ITS DERIVATIVES.

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(*Received May 8th, 1919.*)

Although a clear knowledge of blood pigment and its derivatives is essential to the student, teaching experience has shown how difficult it is to impress so many details upon his mind, and this is mainly because the present nomenclature requires a mnemonic effort on his part. It is perplexing because it does not logically express the relationship between the natural pigment and its derivatives. The reason for this state of things is partly the gradual acquisition of knowledge of these relationships, and partly the arbitrary way in which names were chosen by Hoppe-Seyler, whose authority at the time was sufficiently great to force his nomenclature into the scientific literature of his own and other nations.

Hoppe-Seyler cannot be acquitted of the charge of having ignored, as regards nomenclature, the claims of previous observers. Of this we may take the following examples.

(i) Stokes [1864] in the same paper in which he described the discovery of the spectrum of reduced blood-pigment, proposed on Professor Sharpey's suggestion the name "cruorin" and "reduced cruorin" (scarlet cruorin and purple cruorin). Four years later Hoppe-Seyler [1868] confirmed this discovery in the third part of his full paper on blood pigments. In some previous preliminary communications he had used Berzelius' old name "haematoglobulin[1]," shortened sometimes into "haemoglobin." Under the influence of Stokes' discovery he was obliged to use a second term and employed the somewhat clumsy name "oxyhaemoglobin," promiscuously with haemoglobin, in the second edition of his text-book (1865). He was thus enabled in 1868 to ignore Stokes' nomenclature, especially since Stokes made no further communication on the subject.

(ii) To Thudichum [1867] belongs the credit of having first illustrated and described the spectrum of the pigment obtained by the action of con-

[1] From Liebreich's original manuscript notes of Hoppe-Seyler's lectures in 1863–64 (in the possession of one of us [O.R.]) it is evident that Hoppe, as he was then called, used at that time the term "haematoglobulin" exclusively.

centrated sulphuric acid on blood pigment which he called "cruentine." This fact is not even mentioned by Hoppe-Seyler [1871] when he re-labelled this substance "haematoporphyrin."

(iii) A further instance is the introduction of the term "haemochromogen" to which we shall return directly.

A nomenclature logically expressing the relationship of these substances would start from a short name for the iron-free pigment, and designate its precursors containing iron, protein, and oxygen by suitable prefixes. But an attempt to remodel the nomenclature drastically on such lines (however desirable it would be) would only lead to confusion, for the Hoppe-Seyler nomenclature is at present firmly established in physiological literature. A recent attempt to remodel anatomical nomenclature in a wholesale manner has for corresponding reasons proved to be a failure.

In one point, however, a change is possible, and strongly indicated. The name "haemochromogen," one of the greatest stumbling blocks of the student, should be deleted. The reasons for this are two: first, it is misleading, and secondly it is unnecessary.

It is misleading because the substance is not a "chromogen" in the modern sense, but a coloured substance. The term, therefore, is ill-chosen, and does not in the least indicate its nature.

It is unnecessary because it was adopted to replace another term introduced by its discoverer, and this original name (reduced haematin) has not only priority, but expresses its nature. It was Stokes [1864] who discovered that "brown" alkaline haematin is changed by reducing agents into "red" reduced haematin possessing a characteristic spectrum. Stokes not only discovered the spectrum of reduced haematin, but was also the first to suggest its use for forensic purposes, so characteristic is it, even when the substance is present in minute quantities. It was not until seven years later that Hoppe-Seyler [1871] confirmed Stokes' observation, but gave the substance in question the name of haemochromogen, although he admitted that haemochromogen in alkaline solution is identical with the reduced haematin of Stokes.

The reasons for the adoption of the new term urged by Hoppe-Seyler, are in Gamgee's words [1880]:

"Haemochromogen is a mere product of decomposition of haemoglobin, whilst haematin is an oxidised product of decomposition."

This appears to be a verbal quibble and its justification is entirely lost since even Hoppe-Seyler seems to have dropped his claim of having obtained the substance in crystalline condition directly from haemoglobin [see note by Thierfelder, 1903].

Nearly half a century has elapsed since the introduction of the word haemochromogen, and during this time no evidence has been brought forward to show that reduced haematin and haemochromogen are not one and the same substance. The time has arrived when it is no longer necessary to overburden the nomenclature with a special name. No special name has been found

necessary for reduced haemoglobin; one of us who writes text-books intends in the future to use Stokes' original term, as it is not misleading and expresses the logical relation of the substance to haematin, or, as it will now be necessary to call it, oxy-haematin.

The suggestion is not entirely a new one; Preyer [1871], Linossier [1887], and Abderhalden [1912] have made similar protests.

If this small amendment is made in the nomenclature, we feel sure of its benefit to students, for the relationship of the pigments can then be expressed by the following simple scheme:

Oxy-haemoglobin ↓ *Reducing agents* ↓ Reduced haemoglobin	minus Protein	= Oxy-haematin ↓ *Reducing agents* ↓ = Reduced haematin	=	Haemato-porphyrin and iron

As reducing agents the most frequently employed are either ammonium sulphide or ferrous ammonium tartrate, both originally introduced by Stokes [1864]. Both have disadvantages which it is unnecessary to recapitulate here, for they are well known to those who conduct classes of practical physiology. But perhaps the greatest disadvantage they possess is that, unless very carefully applied, the colour change from arterial to venous blood is not convincing to the junior student. We recommend a solution of sodium hydrosulphite ($Na_2S_2O_4$) as an excellent reducing agent for this purpose. One drop of a 10 per cent. solution is quite sufficient for a test-tube full of dilute oxy-haemoglobin, or oxy-haematin, such as is used in spectroscopic examinations of these substances. The solution itself is colourless, the application of heat is unnecessary, the colour change is immediate and the spectrum is not disturbed by secondary reactions. The employment of this reagent is not new and it seems to have been used about fifty years ago by Schützenberger, its discoverer. He, however, knew the substance only as an unstable solution which had to be prepared from sodium bisulphite by reduction with zinc. The substance is now manufactured on a large scale in this country for the dyeing industry and is obtainable as a dry powder which keeps well. It has the advantage of cheapness over equally satisfactory reducing agents such as hydrazine or hydroxylamine. A 10 per cent. solution of it remains active for many days when kept in a dropping bottle with vaselined stopper.

Summary.

It is suggested that the misleading and unnecessary word haemochromogen be deleted from our vocabulary. Its original name, reduced haematin, should be used instead, and the substance hitherto termed haematin should now be called oxy-haematin.

This would bring the terms into line with reduced and oxy-haemoglobin respectively, and so make the student's work easier in remembering the relationship between blood pigment and its derivatives.

The use of sodium hydrosulphite as a reducing agent is recommended.

REFERENCES.

Abderhalden (1912). *Zeitsch. physiol. Chem.*, **78**, 162.
Gamgee (1880). *Text Book of Physiol. Chem.*, London, **1**, 120.
Hoppe-Seyler (1868). *Med. Chem. Unters.*, Berlin, **3**, 371.
—— (1871). *Med. Chem. Unters.*, Berlin, **4**, 528.
Linossier (1887). *Compt. rend.*, **104**, 1296.
Preyer (1871). *Die Blutkrystalle*, Jena, 200.
Stokes (1864). *Proc. Roy. Soc.*, **13**, 353.
Thierfelder (1903), *Hoppe-Seyler's Handbuch*, footnote to p. 276.
Thudichum (1867). *10th Rep. Med. Off. Privy Council*, 227.

XXII. THE ANTI-SCORBUTIC VALUE OF DRY AND GERMINATED SEEDS.

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(*Received May 13th, 1919.*)

Scurvy is always a menace to any community of people who are separated from adequate supplies of fresh food, whether in tropical or arctic climates. Such it proved to be to our army in Mesopotamia in 1915 and 1916 (*Mesopotamia Commission Report*, 1917), especially among the Indian troops. In consequence of this outbreak, a group of investigators in this Institute undertook an investigation of foodstuffs to determine their relative value as anti-scorbutics and especially to discover some effective material which could easily be transported and would not readily deteriorate. It has been known for centuries by practical experience of human diets, and has recently been proved experimentally [Holst and Frölich, 1912; Delf, 1918] that vegetables of high anti-scorbutic potency become useless for the prevention of scurvy if they are dried and thus rendered suitable for transport.

The most likely solution of the problem had already been indicated by Fürst [1912]. In the series of classical enquiries into experimental scurvy initiated by Holst in the University of Christiania, the work of Fürst showed that anti-scorbutic stuff was developed in substantial quantity during the first days of germination of various seeds. Peas, beans and other pulses, containing as they do only about 10 % of water, are eminently suited for transport, do not readily deteriorate and can be germinated for use where and when required. They form moreover a large part of the normal diet of the Indian soldier.

Experiments at this Institute with guinea-pigs on a diet consisting mainly of peas or lentils confirmed Fürst's results, and these preliminary trials were published in brief by Chick and Hume [1917] in whose paper directions were given for the germination of the dry seeds on active service. The subject appeared, however, to deserve more detailed examination, especially in respect of the anti-scorbutic value of germinated seeds in comparison with other foodstuffs and of its destruction by ordinary methods of cooking. The work undertaken to complete and amplify the earlier trials is set out in the following pages. Protocols of the individual animal experiments are given in the tables, which in many cases contain results of the earlier and later experiments side by side.

I. Experiments of Fürst.

Guinea-pigs fed exclusively on cereals and water, the routine "scurvy diet" in Holst's laboratory, die of acute scurvy in 20 to 30 days. Fürst [1912] showed that when dry pulses were substituted for the cereals, scurvy also occurred, though in a less acute form, and life was to some extent prolonged. Thus five animals on a diet of dry yellow peas and water died of scurvy in 29 to 55 days; on dry green peas three animals out of four showed definite scurvy in 57 to 64 days, while the fourth lived 131 days without signs of the disease. No growth occurred in any of these animals, which indeed lost weight considerably. Fürst's experiments with germinated seeds were made with barley, oats, peas and lentils. These were soaked in water for 24 hours and afterwards kept moist with access of air and allowed to germinate at summer temperature for two or three days, when sprouts were distinctly visible. In this condition the seeds showed distinct anti-scorbutic properties. Thus two animals fed on water and sprouted barley lived 52 and 92 days without any sign of scurvy and four animals on sprouted oats lived 35 to 78 days and only one had symptoms. With peas and lentils even better results were obtained: four animals on germinated green peas lived 143 to 182 days and four on germinated lentils 89 to 183 days without signs of scurvy.

Growth of animals was also defective with germinated seeds, increase of weight taking place only with green peas.

Fürst also made the interesting observation that if germinated barley was dried at 37° the anti-scorbutic properties gained during germination were lost.

II. Methods adopted in the present research.

The method used in this department in the study of experimental scurvy differs from that employed by Holst and his colleagues in two important points. In the first place the uneaten residues of food are collected and weighed each day, so that the actual intake of food is measured as well as the amount of the offered ration; this seems to us essential in any quantitative nutritional experiments. In the second place a daily ration of cow's milk, autoclaved for one hour at 120° C. (to reduce its original content of anti-scorbutic vitamine to a minimum) is in many cases added to the diet to improve its nutritive value without appreciably affecting the supply of antiscorbutic accessory factor.

In the earlier experiments, the basal diet of the guinea-pigs was water with oats and bran *ad libitum*, on which diet death from acute scurvy was found to take place in from 20 to 30 days. To this diet a weighed ration was added daily of the substance whose anti-scorbutic value was to be investigated. When peas or lentils, dry, soaked or germinated, were added to this basal diet, there was little or no growth, irrespective of whether scurvy was or was not prevented. The addition of autoclaved milk, however, helped to supply the missing growth-promoting substances. On a diet of oats, wheaten bran and autoclaved milk, normal growth and development took place if an adequate

amount of the anti-scorbutic factor were supplied in addition. When the supply of the antiscorbutic factor was inadequate, the initial growth was soon checked, scurvy symptoms were observed and death from scurvy occurred in a time only slightly longer than when the diet consisted of grain and water only. (See Table VI.)

The symptoms of guinea-pig scurvy have recently been described in detail [Chick, Hume and Skelton, 1918; Delf, 1918] and it is unnecessary to recapitulate them here.

Dry "Clipper" brand green peas (*Pisum sativum*) and ordinary brown lentils (*Lens esculenta*), unsplit and unhusked, were selected for the experiments. The latter, as "masoor dhal," is a common article of the native Indian diet. Most of the work was done with whole lentils purchased in the ordinary market; they were obtained with difficulty as it is usual to mill, split or crush them before distribution, which practice almost completely destroys their capacity for germination [see Greig, 1917]. Husking of lentils greatly improves their appearance, as the removal of the brown skin leaves a product orange pink in colour and of appetising appearance.

The guinea-pigs used were young growing animals of about 320 to 350 g. weight. They were mostly bred in the Department, to obtain a regular standard of health and vigour.

III. Experiments showing that dry peas have small anti-scorbutic value.

In our experience, young guinea-pigs cannot be induced to consume any considerable amount of either peas or lentils in the dried state. An attempt was made to overcome this difficulty by offering it in the form of meal. The dry peas were ground in a coffee mill and the resulting meal sifted free from the finer particles (which are apt to cake together in a sticky mass when wetted) and shaken free of the skins. The coarse meal thus obtained was moistened with water and allowed to stand for half an hour before being offered to the animals. It was not eaten rapidly, so that some, at least, of the meal was in the soaked condition for some hours before being actually consumed. Residues left over from a day's ration were washed free from particles of the peat on which the animals were bedded, air-dried and weighed.

Three animals were placed upon a diet of oats and bran *ad lib.*, autoclaved milk to 60 cc. and 15 g. dried ground peas (Table I). In no case was this ration of pea-meal eaten regularly and the average amount consumed daily varied from 7 to 10 g. One animal (349) died after 31 days with every symptom of acute scurvy. The other two (347, 348) developed scurvy which became chronic. No. 348 (*B*, fig. 1) consumed a daily average of 9·6 g. pea-meal; after 44 days it became lame, developed loose teeth and had to be fed daily by hand with milk and little pellets of the moistened pea-meal. It continued for a long time in this condition and when killed by chloroform at the end of 105 days was found to be fairly well nourished. There were, however,

Table I. *Experiment with dry peas.*

Number of animal	Ration offered	Average amount consumed daily: Peas g.	Oats and bran g.	Milk cc.	Body weight g.: Initial	Final	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib-junctions	General result
348	15 g. dry pea-meal	9·6	17	52	325	410	Soreness, becoming very lame, joints swollen	105	Bones brittle, rib junctions enlarged, extensive old and recent haemorrhages	Scurvy	Chronic scurvy
349	,, ,,	8·8	20	37	340	315	Soreness, swollen limbs	31	Bones fragile, rib junctions enlarged, muscular and visceral haemorrhages	Severe scurvy	Died of severe scurvy
347	,, ,,	8*	20	40	350	345	Soreness of limbs, scurvy position	30, 59, 15 } 104	Bones brittle, rib junctions ridged, haemorrhages not severe	? Scurvy†	Scurvy

Table II. *Experiments with soaked peas.*

— = not examined.

212	30–40 g. soaked peas	30–40	25 (for 38 days)	30–100 (from 38th day)	375	—	Slight temporary soreness	130	—	—	Good health throughout: returned to stock
214	30–40 g. ,,	28	8	26	310	255	None	128	Bones slightly brittle, rib junctions enlarged, slight haemorrhages, loose teeth	Scurvy	Killed: poor health; slight signs of scurvy
215	30–40 g. ,,	30	24	0	320	318	Limbs tender	40	Slight haemorrhage in heart muscle, teeth slightly brittle	? Scurvy	Death from unknown cause
88	20 g. ,,	28	32	0	320	265	Slight soreness of joints	34	Bones fragile, rib junctions ridged, severe haemorrhages	—	Death from severe scurvy
87	20 g. ,,	19	20	33 (last 32 days)	340	240	Slight soreness of limbs	92	Bones and teeth somewhat brittle	Scurvy	Poor health
208	40 g. ,,	17·5	51	0	500	305	Slight soreness	46	Ribs ridged, teeth loose, haemorrhages	—	Death from scurvy
213	30 g. ,,	18	24	0	358	335	Slight soreness	67	Bones normal, incisors loose, haemorrhages on coecum	? Scurvy	Death from scurvy
91	10 g. ,,	10	30	0	350	240	Soreness of limbs, loose teeth	21	Bones fragile, severe haemorrhages	—	Death from severe scurvy
92	10 g. ,,	10	28	0	380	230	do.	28	Bones fragile, severe haemorrhages	Scurvy	Death from severe scurvy
89	20 g. soaked peas replaced after 38 days by germinated peas	20	33	50 (last 48 days)	330 / 335	335 / 540	Soreness of joints	38, 76 } 114	Normal	Normal	Scurvy cured by germinated peas
90	10 g. soaked peas replaced after 24 days by 30 g. germinated peas	10	29	55 (last 75 days)	335 / 270	270 / 568	Soreness of limbs, loose teeth	24, 84 } 108	—	—	Returned to stock in good health; scurvy cured by germinated peas

* For 30 days, pea-meal then replaced by tamarind juice for 59 days.

† Here and in the other tables "? scurvy" indicates that the rib junctions were not normal, and in most cases they showed the changes which have been previously described as "incipient scurvy" [Tozer, 1918]: as there mentioned, however, it is doubtful whether these alterations indicate anything more than some imperfection in the supply of the accessory substances concerned with growth. They cannot be taken as necessarily indicating scurvy but they are certainly compatible with early or mild scurvy and have to be considered along with the symptoms and other post-mortem signs in estimating the effect of scorbutic diets.

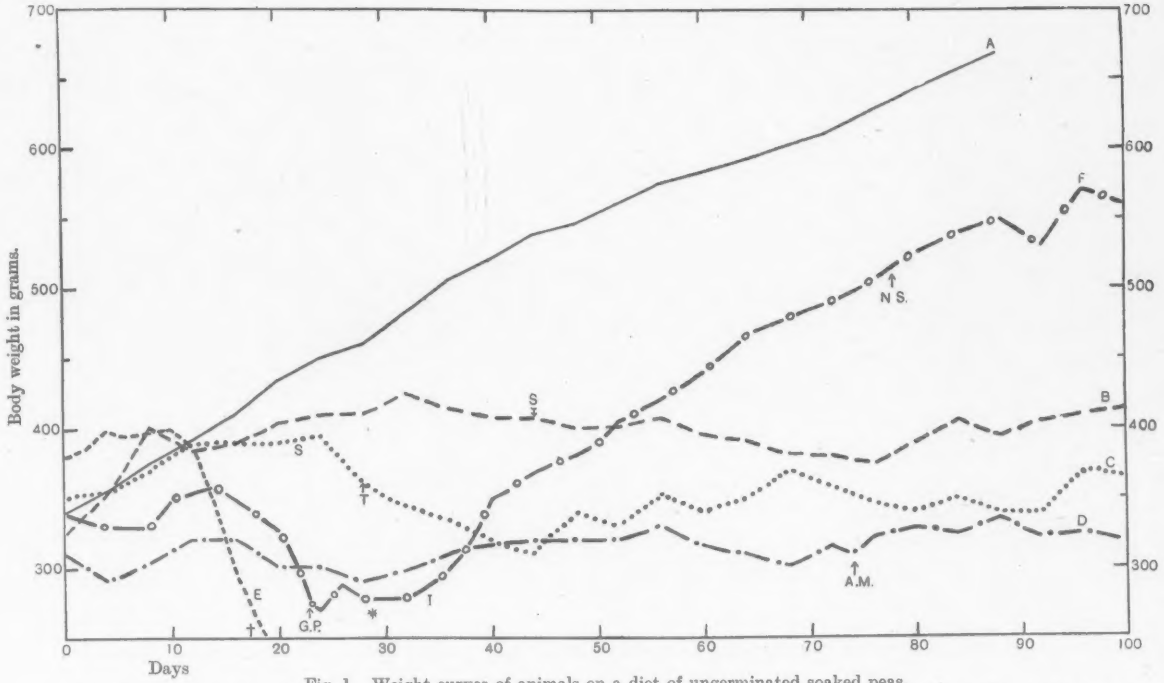


Fig. 1. Weight curves of animals on a diet of ungerminated soaked peas.

A. Control normal weight curve on 30 g. fresh cabbage, oats, bran, and water. *B*. Curve of No. 348 on average daily ration of 9·6 g. pea-meal, 52 cc. autoclaved milk, and oats and bran *ad libitum*. (Tab. I.) Symptoms of severe scurvy were first noted at *S*. *C*. Curve of No. 347 on average daily ration of 8 g. pea-meal, 40 cc. autoclaved milk, oats and bran *ad libitum* for first 30 days. Symptoms of scurvy were first seen at *S*; after 30 days the pea-meal was replaced by tamarind juice and after 89 days both pea-meal and tamarind juice were given daily. *D*. Curve of No. 214 on an average daily ration of 28 g. soaked peas: at *A.M.*, after 74 days, an average of 26 cc. autoclaved milk was taken daily. *E*. Curve of No. 91 on an average daily ration of 10 g. soaked peas (died with severe scurvy). *F*. Curve of No. 90 on an average daily ration of 10 g. soaked peas. After 24 days (at *G.P.*) the animal shewed all the symptoms of severe scurvy, and the soaked peas were replaced by 10 g. germinated peas, and after 28 days (at *) the ration was increased to 30 g. After 34 days (at *I*) there was marked improvement, and 60 cc. autoclaved milk was added to the diet: growth at once began and after 78 days (at *N.S.*) no further symptoms of scurvy could be detected.

extensive haemorrhages in the muscles both recent and of old standing, the bones were fragile and the rib junctions deformed. No. 347 (*C*, fig. 1) developed swollen and painful joints after 21 days and after 30 days was very lame. Its ration of pea-meal, of which it had eaten about 8 g. daily, was then discontinued and its place taken by a daily ration of 15 to 20 cc. of the juice expressed from dried tamarind, previously swelled in water; the rest of the diet remained as before. It lived 59 days on the tamarind juice without showing much change, further progress of the disease being arrested. It was then given pea-meal in addition, no improvement resulted and the animal was chloroformed after a further period of 15 days. The subsequent post-mortem and histological examinations indicated a condition of chronic scurvy.

It is evident from these results that some small degree of protection from scurvy may be derived from a daily dose of 8 to 10 g. of moistened pea-meal. In comparison with other anti-scorbutic materials it has, however, very little potency. Taking, as is our custom, fresh cabbage leaves as the standard, Delf [1918] has shown that young guinea-pigs can be protected from scurvy and maintained in satisfactory health on a diet of oats, bran, autoclaved milk and 1·5 g. fresh cabbage daily: with 0·5 g. definite symptoms of scurvy appear, varying in intensity in different guinea-pigs. It is evident, therefore, that an average daily ration of 9 g. pea-meal has a protective value less than that of 1·5 g. and about equivalent to that of 0·5 g. fresh cabbage: in other words, cabbage is about 15 times as effective as dry peas. The water content of dry peas is about 12 %, of cabbage about 90 %, so that on the dry weights 8 g. of peas are about equal to 0·05 g. cabbage, a ratio of 160 to 1.

IV. Experiments showing that peas and lentils, after soaking in water for 24 hours, have little anti-scorbutic value.

During the process of soaking, the peas and lentils absorbed roughly their own weight of water and the soaked seeds contained 50 to 55 % water, estimated by drying at 100 to 110°. It was difficult to coax the animals to eat large rations; in one case (No. 212), 30 to 40 g. daily were taken; in general, however, 15 to 20 g. soaked peas and 10 to 15 g. lentils was the limit that was voluntarily consumed. Lentils were eaten without preparation, but it was necessary to skin the peas before the guinea-pigs would eat them; this was done after the ration had been weighed out and involved a loss of about 10 % in weight.

In Table II are given the results of experiments with dry green peas after soaking 24 hours in water at room temperature. Scurvy was prevented when a daily ration of 30 to 40 g. was consumed; with 15 to 30 g., scurvy occurred and although two animals out of six survived the three months of the experiment, they had poor health and made little or no growth. When the ration was reduced to 10 g. death from scurvy occurred as in control animals

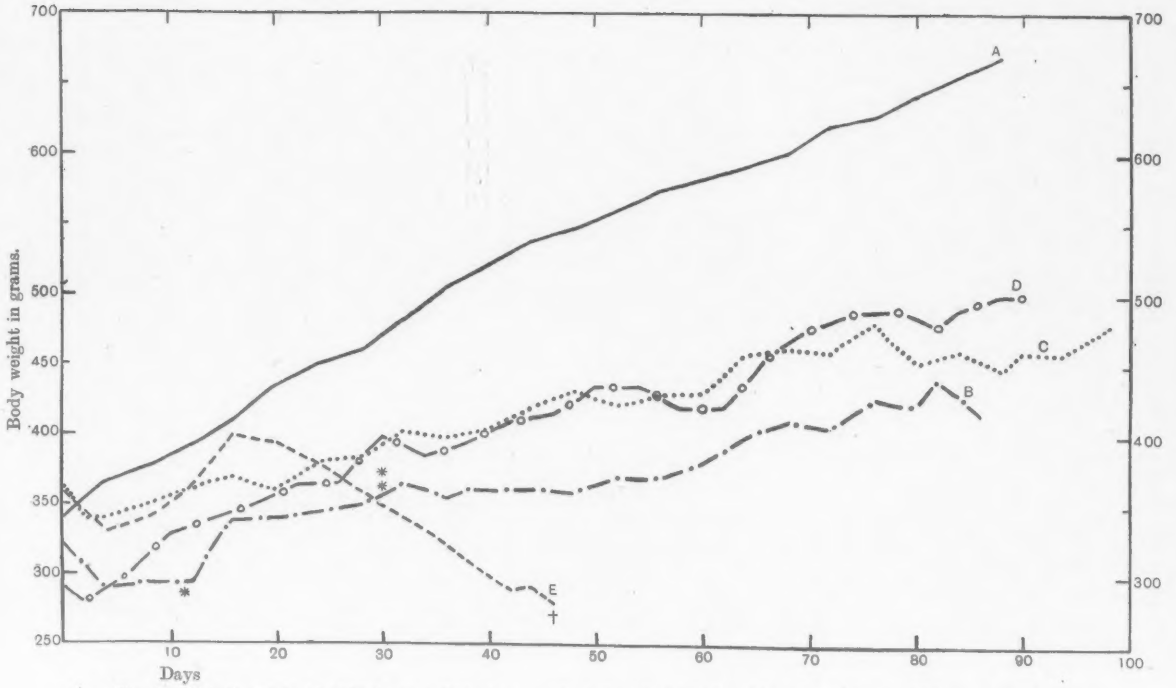


Fig. 2. Weight curves of animals on a diet of ungerminated soaked lentils with oats and bran *ad libitum* in each case.

A. Control normal weight curve on 30 g. raw cabbage, oats, bran and water. *B*. No. 409, on an average ration of 25 g. soaked lentils daily. At * 30 cc., and at ‡ 60 cc. autoclaved milk was added to the diet. *C*. No. 415 on an average ration of 18 g. soaked lentils. From the first, 30 cc. and after 12 days 60 cc. autoclaved milk was included in the diet. Symptoms of scurvy were first observed after 68 days. *D*. No. 412 on an average ration of 15 g. soaked lentils. For the first 20 days 30 cc. and afterwards 60 cc. autoclaved milk was given daily. *E*. No. 413 on an average ration of 10 g. soaked lentils daily. After 4 days 30 cc. and after 26 days 60 cc. autoclaved milk was added to the diet.

(*e.g.* 91 and 92, which died from scurvy in 21 and 28 days). About half the animals received no milk, but the result as regards scurvy was not apparently affected by this.

The case of 89 is interesting. Scurvy, developing on a 20 g. ration of soaked peas, was cured when this ration was substituted by an equivalent weight of germinated peas. Guinea-pig 90 was cured in the same way.

These experiments afford no evidence that soaked peas are better than dry peas. The dry substance of 9 g. dry peas is about equal to that of 18 g. soaked peas, and the effect of the two rations is not markedly different.

Soaked lentils (Table III) have an anti-scorbutic value which is slightly greater than that of soaked peas. Scurvy was prevented with a daily consumption of about 25 g.: with 15 to 18 g. scurvy was apparent but not severe, and 412 and 415 survived the three months of the experiment and put on weight. On a daily consumption of about 9 g., death from severe scurvy occurred in 45 days with much loss of weight. All the animals received autoclaved milk.

In comparing the value of peas and lentils, it should be noted that 10 g. of peas have an average of 30 seeds, and 10 g. of lentils have about 100 seeds.

V. Experiments showing that germinated peas and lentils possess considerable anti-scorbutic value.

The peas and lentils after soaking in water for 24 hours were kept moist with access of air (conveniently in a large funnel) for about 48 hours at room temperature. The radicle was then usually about 1 cm. in length [see the figures in the paper by Chick and Hume, 1917]. The guinea-pigs ate the germinated seeds more readily than when they were merely soaked, though they seemed unable to take more than about 15 g. of lentils daily, whether raw or cooked: with peas, 20 to 30 g. were eaten daily for two or three months without ill effect.

Twenty-four animals received varying amounts of germinated peas (Table V). With a daily consumption of 10 g. and over there was satisfactory protection except in the case of one animal (315) out of ten. Out of six animals on a 5 g. ration, two died of some infection: four lived through the three months of the experiment; these grew fairly well and clearly received considerable protection, although they showed some evidence of scurvy when they were killed for examination. On 2·5 g. two died of acute scurvy in 37 and 53 days respectively and though the other two survived to the end of the experiment, both had the disease in a moderate degree. Four animals which ate daily 7 to 10 g. of peas germinated in the dark were also well protected.

With regard to the growth of these animals, it should be noted that most of those which had the larger pea rations were started with peas, oats and bran, but without milk. On this diet little growth was made. The addition

Table III. *Experiments with soaked lentils.*

Number of animal	Ration offered	Average amount consumed daily: Lentils g.	Oats and bran g.	Milk cc.	Body weight, g.: Initial	Final	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib junctions	General result
409	30 g. soaked lentils	25	27	39	320	415	Sore limbs	87	Normal	Normal	Death, diarrhoea, no scurvy
416	30 g. ,,	23	17	47	345	440	None	54	Jaws fragile, haemorrhage in lungs	Nearly normal	Death from unknown cause, slight scurvy
415	20 g. ,,	18	19	44	358	480	Soreness of limbs, scurvy position	94	Bones normal, slight haemorrhages	Scurvy	Definite chronic scurvy
412	20 g. ,,	15	16	48	290	500	Slight soreness of limbs	90	Normal	Nearly normal	Normal
413	10 g. ,,	10	19	31	362	292	General soreness becoming acute	45	Bones papery, jaws and teeth brittle, slight haemorrhages	Severe scurvy	Death from severe scurvy
414	10 g. ,,	8	18	33	340	301	Limbs sore, joints swollen	45	Bones soft, knees fractured and swollen, haemorrhages severe	Severe scurvy	Death from severe scurvy

Table IV. *Experiment with germinated lentils.*

404	10 to 20 g. germ. lentils	12·2	18	47 (after 30 days)	330	438	None	90	Normal	Normal	Complete protection
405	10 ,, ,,	7·7	39	50 (after 30 days)	358	640	,,	91	,,	,,	,,
407	10 ,, ,,	9·5	28	50 (after 30 days)	330	620	,,	93	,,	,,	,,
411	10 ,, ,,	15·1	16	46 (after 30 days)	320	570	,,	94	,,	,,	,,
427	5 g. ,,	5	26	57	302	540	,,	90	Bones brittle	Severe chronic scurvy	Chronic scurvy, partial protection
428 A	5 g. ,,	5	49	42	342	465	,,	90	Normal	Chronic scurvy	Protection, signs of scurvy in histology only
829	5 g. ,,	5	17	56	320	275	General soreness	51	Fragile bones, severe haemorrhages	Severe scurvy	Death from severe scurvy
830	5 g. ,,	5	25	57	333	355	Kneesmuch swollen	73	do. do.	,,	Death from scurvy
429	2·5 g. ,,	2·5	35	47	318	330	Knees sore	74	Brittle bones and teeth, haemorrhage in stomach, signs of infection	Scurvy	Death from infection, scurvy also
430	2·5 g. ,,	2·5	27	55	310	362	General soreness	78	Bones and teeth brittle, haemorrhage in one knee and coecum, signs of infection	Severe scurvy	do. do.
431	2·5 g. ,,	2·5	62	51	330	375	Limbs sore	70	Teeth brittle, two rib junctions ridged, signs of infection	Scurvy	do. do.
817	2·5 g. ,,	2·5	30	56	370	393	Limbs sore, very lame	90	Bones hard, knees swollen, severe haemorrhages, 3 rib junctions ridged	,,	Severe chronic scurvy
839	2·5 g. ,,	2·5	15	57	345	337	Lame, limbs sore	92	Bones very fragile, rib junctions ridged, severe haemorrhage	Chronic scurvy	,, ,,
818	2·5 g. ,,	2·5	28	54	348	355	Lame, scurvy position	48	Bones fragile, haemorrhages, pneumonia	? Scurvy	Death, from bronchopneumonia, scurvy also

of autoclaved milk at once initiated growth (312, Fig. 3, *B*): autoclaved skimmed milk acted in the same way though less effectively (308, Fig. 3, *C*): with tinned milk (315, Fig. 3, *D*) there was a short period of improvement but the animal afterwards lost weight, refused food and died with diarrhoea.

These scanty data suggest that germinated peas are deficient in the fat-soluble growth factor of which whole milk seems to be the best source among

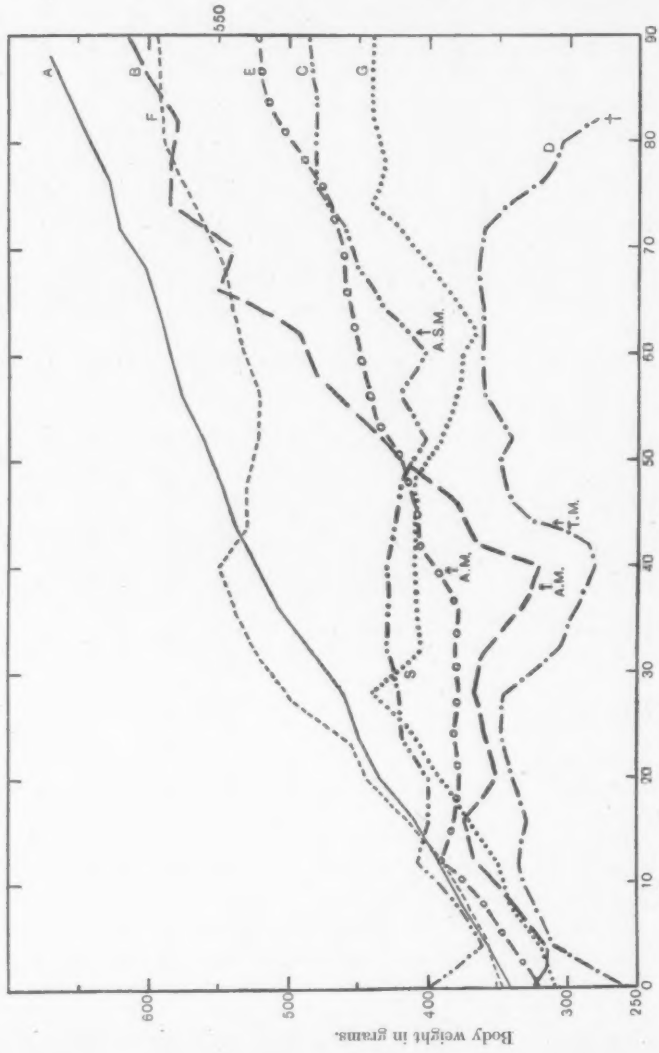


Fig. 3. Weight curves of animals on a diet of germinated peas.

A. Normal weight curve on 30 g. fresh cabbage, oats, bran and water. *B*. No. 312 on an average daily ration of 20–30 g. germinated peas. After 38 days (at *A.M.*) 60 cc. autoclaved milk was added to the diet. *C*. No. 308 on a daily ration of 20 g. germinated peas. After 62 days (at *A.S.M.*) autoclaved skimmed milk was added to the diet. *D*. No. 315 on an average daily ration of 30 to 12 g. germinating peas. After 44 days (at *T.M.*) tinned milk was added to the diet, but the appetite for peas diminished to about 2 g. daily, and scurvy symptoms appeared. *E*. No. 310 on average daily ration of 10 g. germinated peas. After 38 days (at *A.M.*) autoclaved milk was added to the diet. *F*. No. 337 on a daily ration of 5 g. germinated peas with 60 cc. autoclaved milk from the first. *G*. No. 437 on a daily ration of 2·5 g. germinated peas with 60 cc. autoclaved milk from the first. After 30 days, at *S*, symptoms of scurvy first developed.

the different types of milk tried. Before this conclusion could be accepted as an established fact, it would be necessary to show that the diet was adequate as regards all *other* components, to a deficiency of which growth limitation could be attributed—such as the nature and quantity of (1) protein, (2) mineral salts, and (3) water-soluble growth factor. No. 3 is provided in abundance in a diet consisting of peas, oats, and wheaten bran, and it is probable that a possible defect in any of these constituents as regards (1) or (2) will be made good by admixture with the others. The fact that in all these experiments without milk, weight was satisfactorily *maintained* is in favour of this conclusion. It is unfortunate that the point could not be quickly settled by observing whether normal growth takes place on addition to the diet of a small proportion of butter fat, cod liver oil, or other source of the fat-soluble growth factor—the guinea-pig being unable to tolerate pure fat given in this form.

In case of the animals with the smaller rations of peas, 60 cc. of autoclaved milk was offered daily throughout the experiment and was as a rule well taken; in the absence of severe scurvy growth was made (Fig. 3, compare *F* and *G*). It seems, therefore, that a daily ration of 10 g. germinated peas with milk will prevent scurvy and assure good health and growth. The cure of two animals from scurvy by germinated peas has already been mentioned (see Table II).

With germinated lentils (Table IV) complete protection was obtained with a daily consumption of about 10 g. and partial protection with 5 g., while with 2·5 g. definite symptoms developed in every case. In all cases life was substantially prolonged, even when scurvy was not prevented.

With 10 g. of lentils no growth was made without milk but when autoclaved milk was added to the oats and bran, the weight rapidly increased (Fig. 4, *B*, after 30 days). The 2·5 and 5 g. series had milk from the start and made good growth until symptoms of scurvy appeared. The history of the guinea-pigs with the smallest lentil rations was, however, unfortunately obscured by four of the six animals dying of intercurrent disease as is often the case when the diet contains only minimal amounts of an essential accessory factor.

The results with germinated lentils are thus in accord with those for peas: in both cases a 5 g. daily ration seems to be just below, and 10 g. well above, the minimum required for protection.

In Table VI are summarised the results of the foregoing experiments with peas and lentils. In the dry condition peas show traces of anti-scorbutic properties as evidenced by prolongation of life although scurvy is not prevented; the same result was obtained by Fürst [1912]. When soaked in water for 24 hours at room temperature scurvy was prevented with a large daily ration, 30 to 40 g. peas (dry weight, 15 to 20 g.) or 20 to 30 g. lentils (dry weight, 10 to 15 g.). The data do not permit one to decide whether any anti-scorbutic virtue is created during the process of soaking in water, for in the case of the

Table V. *Experiment with germinated peas.*

— = not examined.

Number of animal	Ration offered	Average amount consumed daily: Peas g.	Oats and bran g.	Milk cc.	Body weight: Initial	Final	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib junctions	General result
312	30 g. germinated peas	25	28	50 (after 36 days)	330	620	All the animals on 10 g. peas and upwards shewed signs of soreness of limbs during life unaccompanied, however, by symptoms of scurvy, excepting in one case (No. 315)	93	Normal	Normal	Protection, no scurvy
323	30 g. „	28	25	40 (after 26 days)	410	51		83	—	—	Good health, returned to stock
314	30 g. „	21	25	40 (after 29 days)	270	515		88	—	—	do. do.
315	30 g. „	30 to 12	24	47 (after 44 days)	260	270		75	Fragile bones, loose teeth, muscular and intestinal haemorrhages	—	Death from diarrhoea, scurvy also
308	20 g. „	20	28	50 (after 60 days)	400	495		93	—	—	Good health, returned to stock
309	20 g. „	18	29	52 (after 38 days)	305	550		93	Normal	Normal	Protection, no scurvy
325	15 g. „	14·5	25	48	420	600		84	—	—	Good health, returned to stock
311	10 g. „	10	28	52	320	515		94	Rib junctions slightly ridged	Normal	Protection, no scurvy
313	10 g. „	10	30	47	290	392		69	Bones somewhat fragile, haemorrhages only in ileum and duodenum	Normal	Died from unknown cause, ? any scurvy
310	10 g. „	10	27	52 (after 38 days)	320	535		93	Rib junctions slightly ridged	? Scurvy	Protection
327	5 g. „	5	37	53	345	525	Slight soreness persistent	94	Rib junctions slightly thickened	Severe scurvy	Scurvy
328	5 g. „	4·1	25 (after 68 days)	39 (after 68 days)	305	435	Slight soreness persistent	87	Rib junctions slightly ridged, muscles very dark	? Scurvy	Protection
337	5 g. „	5	26	45	345	560	Scurvy position	97	Rib junctions slightly enlarged, slight haemorrhages	Scurvy	Slight scurvy
341	5 g. „	5	28	44	350	565	Slight soreness	92	do. do.	Normal	Slight scurvy
434	5 g. „	5	29	45	330	360	None	29	Slight subcutaneous haemorrhage	Severe scurvy	Death from infection, also scurvy
435	5 g. „	5	47	55	330	378	Soreness persistent	62	Spleen diseased	do.	do. do.
436	2·5 g. „	2·5	34	46	343	272	Loss in weight	53	Bones very fragile, severe haemorrhages	do.	Death from severe scurvy
437	2·5 g. „	2·5	35	59	309	433	Joints swollen and tender	94	Muscular haemorrhages partly absorbed	Scurvy	Chronic scurvy
438	2·5 g. „	2·3	22	51	330	250	Soreness, scurvy position	37	Bones very fragile, teeth loose and brittle, haemorrhages	Scurvy	Death from severe scurvy
439	2·5 g. „	2·5	46	56	325	645	Soreness lasting 32 days	90	Rib junctions slightly enlarged, teeth loose, jaw brittle	? Scurvy	Slight scurvy
342	10 g. peas germinated in darkness	7	22	57	328	515	Slight soreness	94	Normal	Normal	Protection
343	10 g. do.	10	24	52	340	642	Slight soreness lasting 35 days	102	—	—	Good health, returned to stock
344	10 g. do.	9·5	21	51	313	480	Scurvy position	94	No evidence of scurvy	Normal	Protection
345	10 g. do.	9	28	49	340	450	None	58	do. do.	? Scurvy	Death from intestinal obstruction

pea-meal it was not possible to induce the animals to take a ration larger than 10 g. (dry weight, 9 g.), but the change, if any, cannot be large.

After germination, however, the story is quite otherwise; scurvy was prevented successfully in case of both lentils and peas with a daily ration of 10 g. (dry weight, 5 g.); in many cases 5 g. daily (dry weight, 2·5 g.) was found to be sufficient, while with 2·5 g. daily (dry weight, 1·2 g.) some degree of protection was obtained.

Table VI.

Anti-scorbutic material	Daily ration, g.	Water content, % (approx.)	Dry weight, g.	No. of animals	Time of experiment, days	Result
Peas						
Pea-meal, moistened	8–10	12	7–8·8	3	31–105	Scurvy, but some degree of protection
Soaked 24 hours ...	30–40	50	15–20	2*	128–130	No scurvy
	20	50	10	4	34–114	Scurvy, but distinct protection
	10	50	5	2	21–28	Death from scurvy
Germinated	20–30	50	10–15	6	75–93	Good health in 5 cases
	10	50	5	3	69–94	No scurvy
	5	50	2·5	5†	87–97	Signs of scurvy, but good health in 4 cases
	2·5	50	1·2	4	37–94	Scurvy in all cases, but some degree of protection
Lentils						
Soaked 24 hours ...	30	50	15	2	54–87	No scurvy, protection
	20	50	10	2	90–94	Signs of scurvy but considerable protection
	10	50	5	2	45	Death from scurvy
Germinated	10	50	5	4	91–94	No scurvy, protection
	5	50	2·5	4	51–90	Scurvy in 2 cases, but considerable degree of protection
	2·5	50	1·2	6	48–90	Scurvy, but considerable protection
Fresh lemon juice ...	1·5 cc.	—	—	6	74–90	No scurvy, protection
Fresh orange juice ...	1·5 cc.	—	—	5	62–91	,, ,,
Fresh cabbage leaves	1·5 g.	90	—	5	90	,, ,,
Fresh swede juice ...	2·5 cc.	—	—	8	36–90	,, ,,
Fresh carrot juice ...	20–30 cc.	—	—	8	27–94	,, ,,
Fresh beetroot juice ...	20 cc.	—	—	3	47–90	Scurvy
Fresh green beans ...	5 g.	90	—	4	—	Protection, no scurvy
Control A‡	—	—	—	4	23–30	Death from scurvy
Control B‡	—	—	—	4	34–40	,, ,,

* Omitting No. 215 which died from other causes.

† Omitting 434 and 435 which suffered from an infection.

‡ In case of A the diet consisted of oats, bran and water only; in case of B autoclaved milk 60 cc. was added to the diet.

The anti-scorbutic value of the soaked seeds is thus seen to be increased 3- to 6-fold after 48 hours of germination at room temperature and becomes comparable with that of many fresh vegetables. While distinctly less than that of raw cabbage (minimum protective dose for guinea-pigs 1·5 g. [Delf, 1918]), swede (minimum protective ration 2·5 g. [Chick and Rhodes, 1918]), or fresh orange or lemon juice (minimum protective dose 1·5 cc. [see Chick, Hume, Skelton and Smith 1918]), it is equal to that of raw green (runner)

beans (minimum dose 5 g., unpublished results, Chick and Campbell) and superior to that of raw carrot (minimum dose 20 to 30 g.) or raw beetroot (minimum protective dose more than 20 g. [see Chick and Rhodes 1918]).

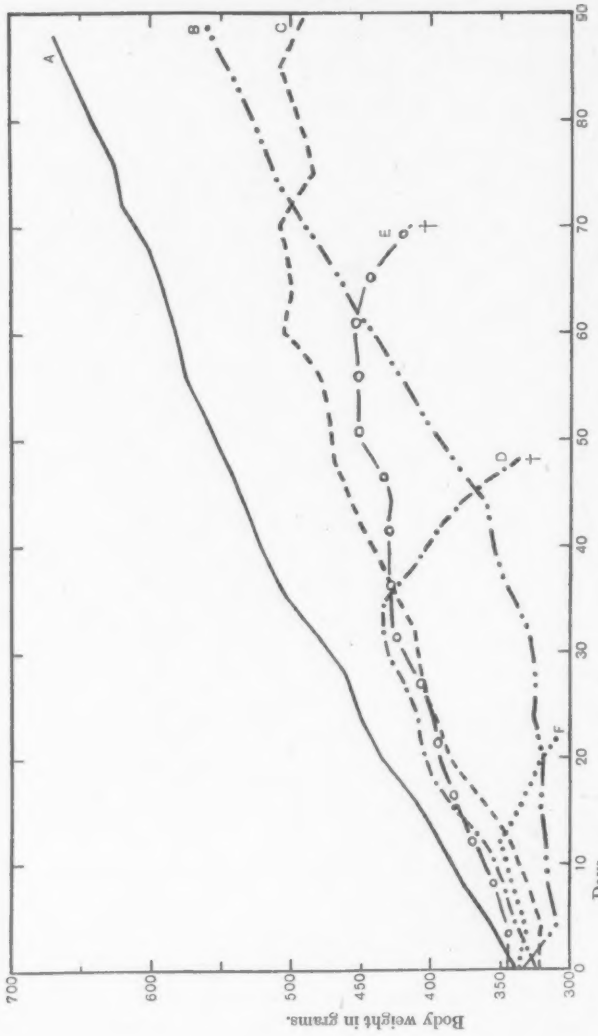


Fig. 4. Weight curves of animals on a diet of raw germinated lentils, oats, bran, and autoclaved milk.

A. Normal weight curve on 30 g. raw cabbage, oats, bran and water. *B.* Mean weight curve of four animals (Nos. 404, 405, 407, 411, Tab. IV) on 10 g. germinated lentils. *C.* Mean weight curve of 427, 428 ♂, the two best animals on 5 g. germinated lentils and 60 cc. autoclaved milk daily. No symptoms of scurvy were seen during life. *D.* Mean curve of 829, 830, the remaining two animals on 5 g. germinated lentils and 60 cc. autoclaved milk daily. *E.* Mean weight curve of Nos. 429, 430, 431, 817, 839, on 2·5 g. raw germinated lentils. At † No. 431 died. *F.* Mean weight curve of Nos. 819, 820, 821 (Tab. VII) on ration of 10 g. germinated lentils boiled in water half an hour, and 60 cc. autoclaved milk.

VI. Experiments with cooked germinated lentils.

Animals were selected which ate raw lentils with relish and amongst these a further selection was made of those which would eat them readily after cooking. It was found (Table VII) that a ration of 10 g. boiled for half or one hour in water was insufficient to protect from severe scurvy. The animals did well for the first two weeks, they put on weight (Fig. 5, *D*), but soon suffered from swollen and sore joints and, when chloroformed, were all found to be suffering from severe scurvy. These animals all received a milk ration.

Five animals were next given a larger ration, 15 g., boiled in water for only 15 minutes, but it was found impossible to ensure that the whole of the amount was eaten. In all cases life was prolonged. One animal, 852 (Fig. 5, *C*), ate an average of 14 g. daily for 91 days and received considerable protection, but even this case showed swollen knees and lameness during life. The remaining four animals were all severely crippled after 30 to 40 days. Cooking had, therefore, reduced the anti-scorbutic value of about 12 g. of germinated lentils to rather less than that of 2·5 g. of the raw material (see Table IV), *i.e.* a loss of about 75 %. This result is in accord with that already established in the case of fresh cabbage, which has been shown to lose about 70 % of its antiscorbutic value when steamed for 20 minutes at 100° [Delf, 1918].

Major E. D. W. Greig, I.M.S., has suggested that the loss during boiling could be reduced by adding traces of some acid to the cooking water. Small amounts of citric acid have no harmful effects on young guinea-pigs [Delf, 1918]. With cabbage, citric acid accelerates the destruction of antiscorbutic stuff by boiling, and with germinated lentils very similar results have been obtained. The cell-sap of the growing lentils is slightly alkaline to litmus and remains neutral or slightly alkaline after boiling with a 0·005 % solution of citric acid. When the concentration is increased to 0·5 %, however, the washed seeds yield a juice which is distinctly acid to litmus. These two solutions were used respectively as the cooking water for the rations in two sets of experiments (Table VIII), and the results show that there is certainly no advantage, and possibly some disadvantage, in using acidified instead of ordinary tap water.

VII. Wiltshire's experiments on the value of germinated beans in the treatment of human scurvy.

Acting on the suggestion made in the preliminary statement of these results [Chick and Hume, 1917, p. 157], Major H. W. Wiltshire, R.A.M.C., has made a most interesting trial of germinated beans in the treatment of scurvy in Serbian soldiers [1918]. Peas could not be obtained, the available lentils were decorticated and would not germinate, so "haricot beans were...first soaked in clean water for 24 hours, and then placed in tin trays for 48 hours to germinate. Old ration biscuit tins, cut in half longitudinally

Table VII. *Experiment with cooked germinated lentils.*

Number of animal	Ration offered	Average amount consumed daily: Lentils g.	Oats and bran g.	Milk cc.	Body weight g.: Initial	Final	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib junctions	General result
809	10 g. germ. lentils boiled 1 hr in water	9·4	28	54	320	260	Scurvy position, great soreness	25	Bones fragile, haemorrhages	Severe scurvy	Severe scurvy
810	do.	8·3	10	47	325	300	do.	30	,, ,,	,,	,,
811	do.	10·3	15	53	350	353	Scurvy position, lame	30	,, ,,	,,	,,
812	do.	8·2	14	55	322	288	do.	29	,, ,,	,,	,,
819	10 g. germ. lentils boiled ½ hr. in water	9·2	13	44	340	275	Soreness, scurvy position	21	,, ,,	,,	,,
820	do.	6·6	15	47	335	275	do.	26	,, ,,	,,	,,
821	do.	8·5	13	56	340	230	do.	37	,, ,,	,,	,,
822	do.	10·2	17	55	325	400	do.	27	Not examined	Not examined	Severe scurvy, cured with orange juice
846	15 g. germ. lentils boiled 15 min. in water	12·3	12	59	370	350	Sore and much swollen knees, scurvy position	82	Bones fragile, severe haemorrhages	Severe scurvy	Emaciated, lame, partial protection
849	do.	12·1	10	52	325	285	do.	53	do. do.	,,	Death from severe scurvy
850	do.	6·3	11	50	325	235	do.	52	do. do.	Scurvy	,, ,,
851	do.	13·7	13	55	350	315	do.	60	do. do.	Severe scurvy	,, ,,
852	do.	14·2	22	59	360	588	do.	91	Bones firm, old haemorrhages at knees, slight recent haemorrhage	? Scurvy	Partial protection

Table VIII. *Experiment with germinated lentils cooked with citric acid.*

859	15 g. germ. lentils boiled 15 min. in 0·005 % citric acid	12·9	15	59	325	555	Soreness, knees swollen	94	Bones normal, slight muscular haemorrhages about knees	? Scurvy	Slight scurvy
860	do.	13·3	8·5	53	330	300	do.	60	Bones soft, loose teeth, severe haemorrhages	Severe scurvy	Death from severe scurvy
872	do.	12·5	9	57	352	382	None	38	Bones slightly fragile, ribs slightly ridged	Scurvy	Death from cause unknown
873	do.	9·4	6	52	331	270	Knees swollen	36	Bones papery, loose teeth, severe haemorrhages and in one lung	Severe scurvy	Death from severe scurvy
863	15 g. germ. lentils boiled 15 min. in 0·5 % citric acid	11·7	9	55	337	244	Scurvy position	30	Bones fragile, haemorrhages severe	,,	,, ,,
864	do.	12·9	9	57	333	300	Sore, knees swollen, scurvy position	89	Bones fragile, rib-junctions nodular, severe haemorrhages	Severe scurvy	Chronic scurvy
874	do.	15	7	58	320	345	Sore, swollen knees	90	Bones fragile, rib junctions ridged, two ribs broken, severe haemorrhages	,,	,,
875	do.	13·3	5	56	335	270	Sore, swollen knees, became helpless	59	Bones very fragile, rib junctions much swollen, severe haemorrhages	,,	Death from severe scurvy

and freely perforated with holes, were found serviceable for this purpose. They were easy to make, clean to handle, and each half held seven pounds of beans, a day's dose for 28 patients. Since germination takes about 48 hours at 60° F., it can easily be carried out in this country (Serbia) in May, when the mean temperature is 67° F. The whole process is very simple, the only essentials being that the seeds must be kept moist but not shut off from

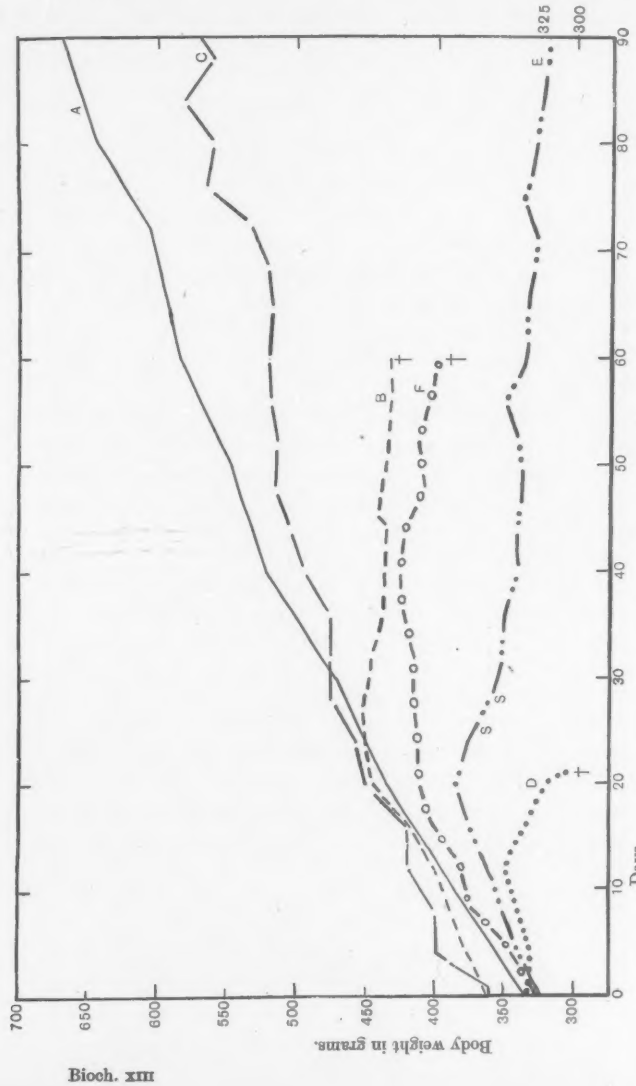


Fig. 5. Weight curves of animals on a diet of cooked germinated lentils, oats, bran and autoclaved milk. *A*. Control normal weight curve on 30 g. raw cabbage, oats, bran and water. *B*. Mean weight curve of 5 animals on diet of 10–15 g. germinated lentils boiled 15 min. in water, oats, bran and 60 cc. autoclaved milk daily: at † No. 852 died. *C*. Weight curve of No. 852, the only animal of this set which escaped scurvy. *D*. Mean weight curve of three animals (819, 820, 821) on diet of 7–9 g. germinated lentils boiled half an hour in water, oats, bran and autoclaved milk. *E*. Mean weight curve of Nos. 864, 874, the two best cases on diet of 13–15 g. germinated lentils boiled 15 min. in solution of 0·5 % citric acid. At *S*, *S*, definite symptoms of scurvy were first seen. *F*. Mean weight curve of Nos. 859, 860, the two best cases on diet of 10–15 g. germinated lentils cooked 15 min. in solution of 0·005 % citric acid. At † No. 860 died.

free circulation of air. After germination the ten minutes' boiling was ample to fit the beans for eating.

"Two wards, each containing 27 beds, were specially devoted to the purpose of comparing the therapeutic values of these germinated beans and fresh lemon juice. Scurvy patients were admitted to these two wards alternately, without selection. In one ward each patient was given four ounces of fresh lemon juice daily, the juice being expressed in the ward, sweetened slightly and given as a measured dose by the sister. In the other ward each patient received a portion of germinated beans which had weighed four ounces in the dry state.

"Thirty patients were treated with lemon juice and twenty-seven with beans. Contrasting them as regards severity of disease at the commencement of treatment, those treated with beans were, on the whole, slightly worse. The difference would be too small to mention, save that it justifies the definite statement that the bean cases were certainly as severe as the lemon cases. Comparing the results of treatment there is a small but definite difference in favour of those treated with beans, 70 % being cured within four weeks as against 53 % of those treated with lemon juice. These figures favour the bean cases unduly, the real difference being better expressed by the (average) time taken for the gums to return to normal, which was 3·1 weeks for bean cases and 3·4 weeks for lemon cases."

Evidently germinated beans are satisfactory for treatment. Whether they are as efficient as fresh lemon juice cannot of course be gauged without further experience with different doses. Experiments on guinea-pigs show (Table VI above) that, as a preventive, lemon juice (but not lime juice: Chick, Hume, Skelton and Smith, *Lancet*, November 30th, 1918) is a good deal superior to germinated peas or lentils.

It is, however, as a *preventive* of scurvy that the inclusion of germinated pulses in a human diet deficient in fresh fruit and vegetables, is principally to be recommended, and so far no definite trials of this type have been reported. In many parts of the world there exists the practice of eating certain seeds in the germinated condition, although there is no suggestion that the anti-scorbutic value of such foods has been appreciated. In the Dutch Indies and Federated Malay States germinated beans or "tow-gay" are eaten raw as a common article of diet [Grijns, 1901; Private communication, Brig.-Gen. Anderson]. In certain districts of China it is the custom to take part of the rice in the germinated condition and especially in the north, beans are artificially sprouted for food in the winter [Report, 1885].

There is little doubt that the anti-scorbutic value of certain fermented beverages, firmly believed in by our ancestors, owed their virtue to the germinated grains from which they were prepared. Captain Cook[1] was a firm believer in the anti-scorbutic properties of a fresh infusion of malt, "sweet-wort." He always took large quantities of malt to sea with him and

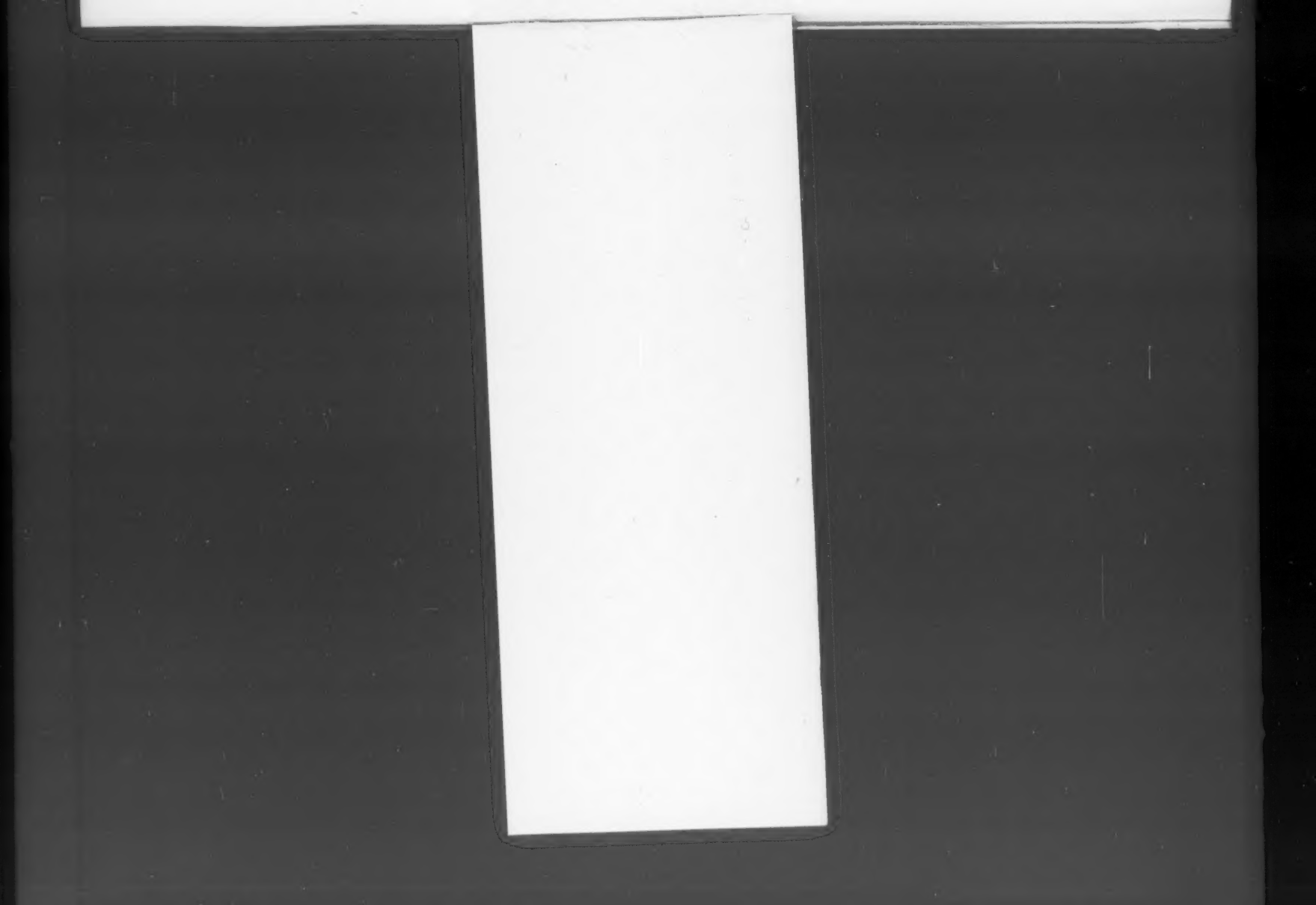
[1] *Captain Cook's Voyages of Discovery.*

ADDENDUM

CHICK and DELF on "The Antiscorbutic Value of Dry and Germinated Seeds," p. 199.

The following note was omitted from p. 217 owing to an oversight:

Note. The histological examinations connected with this investigation were made by Miss F. M. TOZER, to whom we are also indebted for the results given in columns headed "Histology of Rib-Junctions" in Tables I–V, VII and VIII.



the sweet-wort was served out to his men in large quantities when there was any risk of scurvy. "Small beer" also enjoyed a great reputation and in many Arctic expeditions in the first half of the nineteenth century, malt, yeast, and a brewing plant were included in the equipment [see A. Henderson Smith, 1918].

Modern beer prepared from "high dried" malt has been shown by Harden and Zilva [1918] to possess no appreciable anti-scorbutic value and there is no doubt that the virtue these more ancient beverages possessed was due to the freshness of the product, the drink being prepared quickly from freshly germinated grain and consumed shortly after preparation. An interesting incident illustrating this point was reported during the present war by Dyke [1918], in connection with an outbreak of scurvy among a Kaffir labour battalion in France. The diet of this community was arranged to produce as nearly as possible that of the native kraals, where, in spite of a limited diet, scurvy is unknown. The arrangements even included the provision of a native beer brewed from millet, of which large quantities are regularly consumed in Africa. In the enquiry which followed the outbreak of scurvy, the following fact emerged, viz. that the beer as brewed in South Africa was prepared from germinated millet, but that in France the process of germination had been omitted for reasons of convenience.

Summary.

1. Fürst's observation that the content of anti-scorbutic accessory factor in dry peas and lentils is much increased on germination has been confirmed.

2. The antiscorbutic value of these seeds after soaking in water for 24 hours, and germination for 48 hours at room temperature is 5- to 6-fold that of the dry seeds; while inferior to that of orange and lemon juice or cabbages and swedes, it is equal to that of many other vegetables such as green (runner) beans or potatoes, and superior to that of carrot or beetroot. Table VI contains a summary of these results.

3. These seeds, whether in the dry or germinated condition, do not, however, contain a sufficient amount of the growth-promoting substances to induce satisfactory growth in the experimental animals (guinea-pigs) in the absence of milk from the diet. It seems probable that it is the fat-soluble factor that is deficient.

4. A considerable proportion of the anti-scurvy power generated in these germinated seeds is destroyed by boiling: cooking of these germinated seeds should therefore be as short as possible.

5. Wiltshire has shown that germinated beans can be used with success in the treatment of human scurvy. Other evidence is quoted from experience of human diets showing that germinated seeds and their products form valuable material for the prevention of human scurvy.

REFERENCES.

Chick and Hume (1917). *Trans. Soc. Trop. Med. Hyg.*, **10**, 141.
Chick, Hume and Skelton (1918). *Biochem. J.*, **12**, 131.
Chick, Hume, Skelton and Smith (1918). *Lancet*, Nov. 30th.
Chick and Rhodes (1918). *Lancet*, Dec. 7th.
Dyke (1918). *Lancet*, Oct. 19th, p. 513.
Delf (1918). *Biochem. J.*, **12**, 448.
Fürst (1912). *Zeitsch. Hyg.*, **72**, 121.
Greig (1917). *Indian J. Med. Research*, **4**, 818.
Grijns (1901). *Geneesk. Tijdschrift Med. Ind.*, 1.
Harden and Zilva (1918). *J. Inst. Brewing*, **24**, 197.
Holst and Frölich (1912). *Zeitsch. Hyg.*, **72**, 1.
Mesopotamia Commission Report (1917), 10, 71.
Report on Public Health in China (1885).
Smith (1918). *Lancet*, Dec. 14.
Tozer (1918). *Biochem. J.*, **12**, 445, and Plate II.
Wiltshire (1918). *Lancet*, Dec. 14.

XXIII. THE EFFECT OF ALCOHOL ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN.

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(*Received May 19th, 1919.*)

The behaviour of extracts of pancreas or pure pancreatic juice under different conditions has led various observers to conclude that the pancreas contains a number of proteolytic enzymes. It was shown by Fermi [1890] that after treatment with mercuric chloride, salicylic acid and various other substances, trypsin lost its power of digesting fibrin but would still digest gelatin. Vernon [1901] arguing from the varying sensitiveness of pancreatic extracts towards sodium carbonate, concluded that "trypsin" was really a mixture of enzymes of different degrees of stability, the more sensitive enzymes being destroyed first. Vernon only tested the digestive power of trypsin on raw fibrin in this connection however. In a later paper Vernon [1903, 2] states that pancreatic extracts contain an erepsin as well as trypsin. Pollak [1905] using different preparations of enzymes found that the relative amounts of serum and gelatin digested varied enormously in different cases. He also found that after treatment with hydrochloric acid trypsin lost its power of acting on serum, but was still about as active as ever on gelatin. Pollak concluded that extracts of pancreas contained in addition to trypsin (to which the action on serum was due) a special enzyme which acted only on gelatin. To this enzyme Pollak gave the name of glutinase.

According to Ascoli and Neppi [1908], however, this assumption of a special enzyme acting on gelatin is unjustified, as they find that slight variations in the reaction of the medium affect the digestion of different proteins to different degrees. Mays [1906] after a long series of experiments remarks that the presence of two proteolytic enzymes in pancreatic extracts can only be proved when it is possible to make a separation of the enzymes. It had been previously shown by Bayliss and Starling [1903] that pancreatic juice as secreted contains no trypsin (as tested on coagulated egg white), but contains a weak enzyme like erepsin. This has some action on caseinogen, but very slight. The erepsin has a slight action on fresh fibrin but practically none on fibrin which has been heated to 70°. It may here be mentioned that Long and Barton [1914] state that raw fibrin even when very carefully purified may soon become liquid owing to autolysis.

In later papers Fermi [1913, 1914] contests the theory that some proteolytic enzymes have a specific action, and maintains that all proteolytic enzymes have a general action on all proteins.

Slight differences of behaviour of trypsin towards different proteins under the same conditions have also been noted by Berg and Gies [1906], Porter [1910], Long and Hull [1917], but not much importance seems to have been attached to the facts. Others such as Glaessner and Stauber [1910] and Auerbach and Pick [1912] find differences between the proteolytic and peptolytic actions of trypsin, but in these cases possibly some of the action was due to the pancreatic erepsin also.

It seems to have been assumed, however, by all the authors quoted and by others such as Hedin [1905] that trypsin is the enzyme responsible for the digestion of fibrin and caseinogen, especially in experiments lasting only a few hours.

The action of alcohol on trypsin has been variously stated. Fermi and Pernossi [1894] using Mett's tubes filled with gelatin found that in presence of alcohol trypsin had more digestive action than in presence of water only. The percentage of alcohol used is not stated. Chittenden and Mendel [1896] found that the action of trypsin on fibrin was markedly inhibited by alcohol, but did not test the action on any other substrate. Dastre [1896] found that trypsin still digested fibrin and boiled albumin in presence of 15 to 20 per cent. of alcohol, while Gizelt [1906, 1, 2] states that 20 % alcohol totally inhibits trypsin. According to Bayliss [1915] trypsin will digest gliadin even in presence of 80 % alcohol, the action in this case being due to the trypsin in suspension. Vernon [1903, 1] noted that dilute alcohol had a considerable inhibitory effect on the digestion of raw fibrin by trypsin.

As dilute alcohol is frequently used in making extracts of various digestive organs, it is important to know how the digestive action is affected thereby.

Experimental Details.

The experiments were carried out as described previously [Edie, 1914]. Ox fibrin after being finely minced and thoroughly washed was suspended in water and gradually heated to 85°. The fibrin was then pressed dry and preserved in glycerol and a little chloroform until required. The caseinogen was a 3 % solution in 1 % sodium carbonate. The pancreatic extracts were prepared by finely mincing sheep's pancreas and extracting with chloroform water for about a fortnight. The extract was then filtered and a little chloroform added as a preservative.

The digestion was carried on at 37° in small flasks, a small measured quantity of chloroform being added to exclude bacterial action in every case. When fibrin was used, the amount of digestion was estimated by filtering off the undissolved fibrin and determining the nitrogen in the filtrate by a Kjeldahl determination. When caseinogen was the substrate, the amount of

digestion was found by precipitation with tannic acid and subsequent estimation of the nitrogen in the filtrate. Controls showed that the sodium carbonate alone had no digestive action whatever either on fibrin or caseinogen. The following are typical results showing the effect of dilute alcohol on the digestion of fibrin and caseinogen by trypsin.

			Digestion in cc. of $N/10$ nitrogen
1.	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	4·6
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	17·1
		1·4 g. fibrin added. Digestion 3 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	23·8
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	23·6
		Digestion 1 hour.	
2.	(*a*)	1 c.c. trypsin, 20 c.c. 12 % alcohol, 20 c.c. 1 % Na_2CO_3	4·6
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	13·8
		1 g. fibrin. Digestion 2·75 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 12 % alcohol, 20 c.c. caseinogen	24·1
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	24·3
		Digestion 1·25 hours.	
3.	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	3·8
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	14·1
		1·3 g. fibrin. Digestion 2 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	27·9
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	27·0
		Digestion 1·25 hours.	
4.	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	8·1
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23·6
		1·5 g. fibrin. Digestion 2·25 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	30·0
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	29·3
		Digestion 1 hour.	
5.	(*a*)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. 1 % Na_2CO_3	5·1
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	12·1
		1 g. fibrin. Digestion 3 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. caseinogen	30·6
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	30·6
		Digestion 1·25 hours.	
6.	(*a*)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. 1 % Na_2CO_3	4·9
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	12·1
		1 g. fibrin. Digestion 3 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. caseinogen	27·7
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	28·0
		Digestion 1 hour.	
7.	(*a*)	1 c.c. trypsin, 20 c.c. 6 % alcohol, 20 c.c. 1 % Na_2CO_3	11·5
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	20·3
		1 g. fibrin. Digestion 3 hours.	
	(*a*)	1 c.c. trypsin, 20 c.c. 6 % alcohol, 20 c.c. caseinogen	22·2
	(*b*)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	22·0
		Digestion 1 hour.	

					Digestion in cc. of $N/10$ nitrogen
8.	(*a*) 1 c.c. trypsin, 20 c.c. 6 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	12·5
	(*b*) 1 c.c. trypsin, 20 c.c. water, 1 g. fibrin. Digestion 3 hours.	20 c.c. 1 % Na_2CO_3	...	...	22·2
	(*a*) 1 c.c. trypsin, 20 c.c. 6 % alcohol,	20 c.c. caseinogen	...	...	25·0
	(*b*) 1 c.c. trypsin, 20 c.c. water, Digestion 1 hour.	20 c.c. caseinogen	...	...	24·6
9.	(*a*) 5 c.c. trypsin, 20 c.c. 16 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	7·1
	(*b*) 5 c.c. trypsin, 20 c.c. water, 1 g. fibrin. Digestion 2 hours.	20 c.c. 1 % Na_2CO_3	...	...	18·2
	(*a*) 5 c.c. trypsin, 20 c.c. 16 % alcohol,	20 c.c. caseinogen	...	...	31·6
	(*b*) 5 c.c. trypsin, 20 c.c. water, Digestion 1 hour.	20 c.c. caseinogen	...	...	31·9
10.	(*a*) 5 c.c. trypsin, 20 c.c. 13 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	14·0
	(*b*) 5 c.c. trypsin, 20 c.c. water, 1·3 g. fibrin. Digestion 3 hours.	20 c.c. 1 % Na_2CO_3	...	...	25·2
	(*a*) 5 c.c. trypsin, 20 c.c. 13 % alcohol,	20 c.c. caseinogen	...	...	34·5
	(*b*) 5 c.c. trypsin, 20 c.c. water, Digestion 1·25 hours.	20 c.c. caseinogen	...	...	34·6
11.	(*a*) 5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	13·7
	(*b*) 5 c.c. trypsin, 20 c.c. water, 1·2 g. fibrin. Digestion 2·5 hours.	20 c.c. 1 % Na_2CO_3	...	...	26·8
	(*a*) 5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. caseinogen	...	...	32·5
	(*b*) 5 c.c. trypsin, 20 c.c. water, Digestion 1 hour.	20 c.c. caseinogen	...	...	32·5
12.	(*a*) 5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	10·8
	(*b*) 5 c.c. trypsin, 20 c.c. water, 1 g. fibrin. Digestion 2 hours.	20 c.c. 1 % Na_2CO_3	...	...	24·6
	(*a*) 5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. caseinogen	...	...	30·0
	(*b*) 5 c.c. trypsin, 20 c.c. water, Digestion 1 hour.	20 c.c. caseinogen	...	...	30·1

These experiments are sufficient to show that alcohol, when present in percentages varying from 3 to 7, has a very marked inhibitory effect on the digestion of fibrin by trypsin but no such effect on the digestion of caseinogen. The amount of fibrin digested under these conditions varied from about 25 to 50 % of the amount digested in absence of alcohol, the proportion varying somewhat with different trypsin solutions and with varying percentages of alcohol. In no case was there any appreciable difference in the amount of caseinogen digested, beyond the limits of experimental error.

With higher percentages of alcohol the digestion of fibrin was in some cases entirely stopped, a fair amount of caseinogen still being digested, however.

					Digestion in c.c. of $N/10$ nitrogen
13.	(*a*) 1 c.c. trypsin, 20 c.c. 25 % alcohol,	20 c.c. 1 % Na_2CO_3	...	...	3·1
	(*b*) 1 c.c. trypsin, 20 c.c. water, 1 g. fibrin. Digestion 3 hours.	20 c.c. 1 % Na_2CO_3	...	...	27·2
	(*a*) 1 c.c. trypsin, 20 c.c. 25 % alcohol,	20 c.c. caseinogen	...	...	20·0
	(*b*) 1 c.c. trypsin, 20 c.c. water, Digestion 1 hour.	20 c.c. caseinogen	...	...	23·7

		Digestion in cc. of $N/10$ nitrogen
14.	(*a*) 1 c.c. trypsin, 20 c.c. 25 % alcohol, 20 c.c. 1 % Na_2CO_3	4·8
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	25·2
	0·8 g. fibrin. Digestion 3 hours.	
	(*a*) 1 c.c. trypsin, 20 c.c. 25 % alcohol, 20 c.c. caseinogen	32·8
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	37·9
	Digestion 1·75 hours.	
15.	(*a*) 1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. 1 % Na_2CO_3	0·0
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23·6
	1 g. fibrin. Digestion 3 hours.	
	(*a*) 1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. caseinogen	5·3
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	25·2
	Digestion 1 hour.	
16.	(*a*) 1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. 1% Na_2CO_3	0·0
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23·4
	1·2 g. fibrin. Digestion 3 hours.	
	(*a*) 1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. caseinogen	4·6
	(*b*) 1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	28·2
	Digestion 1 hour.	

These experiments show that in presence of 25 % of alcohol the digestion of fibrin by trypsin is entirely inhibited, while digestion of caseinogen still proceeds to a limited extent. In presence of 12 % alcohol the amount of fibrin digested is from 10 to 20 % of the control, while the caseinogen digested amounts to about 85 % of the control.

Trypsin is well known to be very unstable under some circumstances, and it was considered possible that contact with dilute alcohol for some time might lead to an actual destruction of that part of the enzyme molecule which digests fibrin. The following experiments were carried out to test such a theory.

		Digestion in c.c. of $N/10$ nitrogen
17.	(*a*) 20 c.c. trypsin, 15 c.c. 15 % alcohol } kept at 37° C. for 3 hours	
	(*b*) 20 c.c. trypsin, 15 c.c. water }	
	2 c.c. of (*a*), 40 c.c. 0·5 % Na_2CO_3	31·2
	2 c.c. of (*b*), 40 c.c. 0·5 % Na_2CO_3	31·0
	1·3 g. fibrin. Digestion 3 hours.	
18.	(*a*) 20 c.c. trypsin, 5 c.c. 30 % alcohol } kept at 37° C. for 3 hours	
	(*b*) 20 c.c. trypsin, 5 c.c. water }	
	2 c.c. of (*a*), 40 c.c. 0·5 % Na_2CO_3	18·2
	2 c.c. of (*b*), 40 c.c. 0·5 % Na_2CO_3	18·3
	1·2 g. fibrin. Digestion 2 hours.	
19.	(*a*) 40 c.c. trypsin, 10 c.c. 30 % alcohol } kept at 37° C. for 3 hours	
	(*b*) 40 c.c. trypsin, 10 c.c. water }	
	1 c.c. of (*a*), 40 c.c. 0·5 % Na_2CO_3	14·0
	1 c.c. of (*b*), 40 c.c. 0·5 % Na_2CO_3	14·2
	1 g. fibrin. Digestion 3 hours.	
20.	(*a*) 15 c.c. trypsin, 10 c.c. 15 % alcohol } kept at 37° C. for 3 hours	
	(*b*) 15 c.c. trypsin, 10 c.c. water }	
	2 c.c. of (*a*), 40 c.c. 0·5 % Na_2CO_3	17·4
	2 c.c. of (*b*), 40 c.c. 0·5 % Na_2CO_3	17·0
	1 g. fibrin. Digestion 2·75 hours.	

		Digestion in cc. of $N/10$ nitrogen
21.	(*a*) 15 c.c. trypsin, 10 c.c. 15 % alcohol } kept at 37° C. for 3 hours	
	(*b*) 15 c.c. trypsin, 10 c.c. water }	
	2 c.c. of (*a*), 40 c.c. 0·5 % Na_2CO_3	16·3
	2 c.c. of (*b*), 40 c.c. 0·5 % Na_2CO_3	16·2
	1 g. fibrin. Digestion 2·75 hours.	

No destruction whatever of the trypsin is caused by the action of 6 % alcohol, although the digestive action of the enzyme is reduced to 30 % or less of the normal amount by the presence of this proportion of alcohol.

A solid substrate such as fibrin might be rendered less digestible by prolonged treatment with concentrated alcohol, owing to the hardening thus brought about. Alcohol of under 30 %, however, could hardly be supposed to have such an effect, and a few experiments showed that after treatment with dilute alcohol fibrin was no less digestible by trypsin than previously.

		Digestion in c.c. of $N/10$ nitrogen
22.	(1) Fibrin + 10 % alcohol } kept at 37° C. for 3 hours	
	(2) Fibrin + water }	
	1 c.c. trypsin, 40 c.c. 0·5 % Na_2CO_3, 1 g. fibrin (1)	19·3
	1 c.c. trypsin, 40 c.c. 0·5 % Na_2CO_3, 1 g. fibrin (2)	18·1
	Digestion 2·5 hours.	
23.	(1) Fibrin + 10 % alcohol } kept at 37° C. for 19 hours	
	(2) Fibrin + 10 % alcohol }	
	1 c.c. trypsin, 40 c.c. 0·5 % Na_2CO_3, 1 g. fibrin (1)	22·9
	1 c.c. trypsin, 40 c.c. 0·5 % Na_2CO_3, 1 g. fibrin (2)	20·2
	Digestion 3 hours.	

The fibrin which was to be treated with alcohol in these experiments was first washed with alcohol in order to remove any adherent moisture. It will be seen that after treatment with 10 % alcohol fibrin is apparently slightly more readily attacked by trypsin than previously.

The action of trypsin on fibrin and on caseinogen is affected by dilute alcohol to such different degrees that it is reasonable to suppose either that there are two enzymes concerned in the digestion of these proteins or that different groups of the same enzyme molecule take part in the hydrolysis of the different proteins. In the latter case the groups which digest fibrin are very much more easily inhibited by alcohol than the groups which digest caseinogen.

The theory that different side chains in the molecule of an enzyme are responsible for different functions is used to explain the zymoid modification of enzymes. Some observers also, for example, Nencki and Sieber [1901], hold that the behaviour of pepsin and rennin under varying conditions can best be explained on the theory that only one enzyme is concerned here, with different side chains responsible for the proteolytic and milk coagulating functions. Vernon [1903, 1] also considers this probable in the case of the milk coagulating and proteolytic actions of trypsin.

Hitherto it has apparently been assumed that one enzyme "trypsin" is responsible for the digestion of fibrin and caseinogen by pancreatic extracts.

In this case the function is the same (hydrolysis of a protein to form simpler products), but it would seem that different side chains may be necessary for the hydrolysis of different proteins.

SUMMARY.

Alcohol when present to the extent of 3 % and upwards markedly inhibits the action of trypsin on fibrin. The digestion of caseinogen by trypsin is not affected until the concentration reaches 10 %. The action of alcohol is not due to the destruction of the trypsin, since on suitable dilution of the mixture of trypsin and alcohol the digestion of fibrin is as great as in the control.

Fibrin is not rendered less digestible by contact with dilute alcohol, but seems to be slightly more readily dissolved by trypsin than previously.

If "trypsin" is a single enzyme the digestion of fibrin and caseinogen is probably carried on by different side chains, those digesting fibrin being much more readily affected by alcohol than the others.

REFERENCES.

Ascoli and Neppi (1908). *Zeitsch. physiol. Chem.*, **56**, 135.
Auerbach and Pick (1912). Quoted from *Biochem. Centralbl.*, **14**, 1668.
Bayliss (1915). *J. Physiol.*, **50**, 90.
Bayliss and Starling (1903). *J. Physiol.*, **30**, 61.
Berg and Gies (1906). *J. Biol. Chem.*, **2**, 489.
Chittenden and Mendel (1896). *Amer. J. Med. Sci.*, **111**, 181.
Dastre (1896). *Arch. Physiol.* (Ser. 5), **8**, 120.
Edie (1914). *Biochem. J.*, **8**, 193.
Fermi (1890). *Arch. Hygiene*, **10**, 1.
—— (1913). *Centralbl. Bakt. Par.*, I Orig., **68**, 433.
—— (1914). *Centralbl. Bakt. Par.*, I Orig. **72**, 401.
Fermi and Pernossi (1894). *Zeitsch. Hygiene*, **18**, 83.
Gizelt (1906, 1). *Centralbl. Physiol.*, **19**, 769.
—— (1906, 2). *Pfluger's Arch.*, **111**, 620.
Glaessner and Stauber (1910). *Biochem. Zeitsch.*, **25**, 204.
Hedin (1905). *J. Physiol.*, **32**, 468.
Long and Barton (1914). *J. Amer. Chem. Soc.*, **36**, 2151.
Long and Hull (1917). *J. Amer. Chem. Soc.*, **39**, 1051.
Mays (1906). *Zeitsch. physiol. Chem.*, **49**, 124.
Nencki and Sieber (1901). *Zeitsch. physiol. Chem.*, **32**, 291.
Pollak (1905). *Beiträge*, **6**, 95.
Porter (1910). *Quart. J. Exper. Physiol.*, **3**, 375.
Vernon (1901). *J. Physiol.*, **26**, 405.
—— (1903, 1). *J. Physiol.*, **29**, 302.
—— (1903, 2). *J. Physiol.*, **30**, 330.